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13. ABSTRACT (Maximum 200 Words)

THIS IS THE THIRD YEAR OF A FOUR-YEAR PROJECT TO EXPERIMENTALLY ASSESS THE NEUROTOXICOLOGIC POTENTIAL OF EXPOSURE TO DEPLETED URANIUM (DU), AND THE ROLE OF STRESS IN ALTERING THIS. IN THIS YEAR, THE THIRD (OF FOUR) TASKS IN THE STATEMENT OF WORK WAS LARGELY ADDRESSED. THIS WAS THE PERFORMANCE OF AN ACUTE (30 DAY) STUDY OF THE NEUROTOXICITY OF SOLUBLE DEPLETED URANIUM (ADMINISTERED AS URANYL ACETATE) IN RATS. A SINGLE INTRAMUSCULAR DOSE OF 0.1, 0.3 OR 1.0 MG/KG DU WAS ADMINISTERED, SHORTLY AFTER EXPOSURE TO A SEQUENCE OF RESTRAINT AND SWIMMING STRESS THAT RESULTED IN MARKED, TRANSIENT ELEVATION OF PLASMA CORTICOSTERONE. CLINICAL, NEUROCHEMICAL AND PATHOLOGICAL STUDIES WERE UNDERTAKEN. MAJOR FINDINGS WERE TRANSIENT DECREASE IN DOPAMINE IN THE CAUDATE-PUTAMEN ON POST-DOSING DAY 3 IN THE HIGH DOSE, UNSTRESSED ANIMALS. THERE WERE DU-RELATED DECREASES IN MOTOR ACTIVITY, BODY WEIGHT GAIN AND FORELIMB GRIP STRENGTH. DU DOSE-RELATED RENAL TUBULAR NECROSIS WAS ALSO SEEN, AND MAY HAVE CONTRIBUTED TO THESE CLINICAL FINDINGS. STRESS HAD NO EFFECT ON THE RENAL DISEASE OR THE CLINICAL CHANGES, EXCEPT FOR A PROTECTIVE EFFECT IN THE GRIP STRENGTH. TASK 4, A LONG-TERM DU-IMPLANTATION/STRESS STUDY, WAS INITIATED IN THIS YEAR. DATA FROM AN EARLIER TASK, A PILOT STRESS STUDY, REVEALED THAT 6 WEEKS EXPOSURE TO THE RESTRAINT AND SWIM STRESS, OR SWIM ALONE, RESULTED IN REGIONAL NEURONAL DENDRITIC ATROPHY IN THE HIPPOCAMPUS.

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Introduction

This is a four-year study on the neurotoxic potential of depleted uranium (DU) in laboratory rats. Previous studies with Gulf War veterans and experimental animals exposed to embedded DU suggest that neurotoxicity may result from DU exposure (McDiarmid *et al.* 2000; Pellmar *et al.*, 1999). The current investigation is designed to assess the neurotoxic potential of acute and chronic exposure to DU and the contribution of stress to expression of DU neurotoxicity and kinetics. All studies are being performed with adult male Sprague-Dawley rats. As noted in the Table below, the components of this project are as follows:

1- Dose-finding and preliminary toxicokinetic studies of DU. These were conducted in the Year 1 of the project
2- Pilot stress study- To obtain an optimal model of experimental stress for use in the acute and chronic DU exposure studies. This was done in Year 2 and Year 3.
3- Acute neurotoxicity of soluble DU (uranyl acetate)- To determine nervous system kinetics and toxicity of DU. This was a major focus in Year 3. The in-life portion was done, and the neurobehavioral and most of the neurochemical data were obtained and analyzed. Neuropathological studies of the brain were also performed in this year. Tissue uranium assays were done in part.
4- Long-term toxicity study- This will assess chronic exposures from implanted solid DU particles, a model of some Gulf War casualties. This study began in Year 3, and is in progress.

Methods for both the acute and chronic (3, 4 - above) studies employed the following procedures. Inductively coupled plasma-mass spectrometry (ICP-MS) analysis to assess the kinetics of uranium in the cerebral cortex, hippocampus, striatum, and cerebellum at selected periods after DU administration. Neurotoxicity is assessed with behavioral, morphological, and biochemical endpoints. Behavioral assessment of neurotoxicity utilizes the Functional Observation Battery (FOB), motor activity, and tests of learning and memory (by passive/active avoidance). Biochemical analyses include quantification of neurotransmitters (dopamine, norepinephrine, serotonin, glutamate, GABA), determination of receptor number and indicators of oxidative stress (levels of oxidized and reduced glutathione), done in the same brain regions examined for DU kinetics. Morphological studies employ perfusion-fixation, multilevel sampling of the nervous system and contemporary light microscopic procedures to allow detailed evaluation of any lesions. Based upon the above noted pilot stress study, a model of daily restraint with intermittent swimming stress is employed, in association with DU exposure These studies will help define the neurotoxic potential of DU and assess the role of stress in modifying this effect.

This is the annual report for year three of this four year study, the major activities of which involved performance of the acute studies of DU neurotoxicity. The progress of the project is indicated in Table 1.

Table 1. Progress of Award # DAMD17-01-0775, Multifactorial Assessment of Depleted Uranium Neurotoxicity as of September 30, 2004 (end of Year 3)

Task	**Status**
1. Preliminary studies for dose range finding for acute and chronic studies	complete
2. Toxicokinetic study: a. Determination of serum and regional brain uranium concentrations b. Determine the effects of stress on DU kinetics	complete
3. Pilot stress study	complete
4. Acute toxicity study	In-life completed, all in-life and post-mortem analyses completed, except for some uranium and neurochemical assays, and part of the neuropathology
5. Long-term toxicity study	In progress (in-life to be completed June, 2005)

Body

I. Tasks from Statement of Work (as revised 1/10/03) and Accomplishments- This is a complex, four-year study on the neurotoxic potential of depleted uranium and its modulation by stress. What follows are tasks described in the approved Statement of Work (see Appendix), along with relevant research accomplishments for Year 3.

A. ***Task 1***- Perform **preliminary studies** to identify appropriate doses of DU for kinetic studies.

   Research Accomplishments for Task 1. This has been completed, and reported in the October 2002 and 2003 Annual Reports.

B. ***Task 2***- Perform a **toxicokinetic study**. This is to determine the concentration of DU in rat serum, brain cortex, hippocampus, striatum, and cerebellum at several times after DU exposure. The effect of stress on DU kinetics will also be determined.

   Research Accomplishments for Task 2. This has been completed, and reported in the October, 2002 Annual Report. A paper reporting these results is in press in the Journal of Toxicology and Environmental Health (see Barber *et al.*, J. Toxicol. Environ. Health, in Reportable Outcomes and Appendix, below).

C. ***Task 3***- Perform a **pilot stress study,** to determine the most appropriate model of stress to employ in subsequent acute and long-term DU neurotoxicity studies. This was done by comparing several stress models in Sprague-Dawley rats, to determine the best way to obtain peak plasma corticosterone level at the time of DU exposure. This was largely completed in Year 2, and reported in the October, 2003 Annual Report. Some additional data is reported here, along with a review to provide some perspective. The stress models were (all administered stress 5 days/week for 6 weeks, n=8/group): Group 1- handling (control); Group 2- restraint for 30 minutes; Group 3- 4 days restraint for 30 minutes, followed by 1 day swimming for 30 minutes; Group 4- swimming for 30 minutes. Results indicated that corticosterone levels were elevated in animals stressed by restraint for 4 days (Mon-Thurs) and swimming on day 5 (Friday) (Figure 1). Plasma corticosterone levels in Groups 3 (restraint/swim) and 4 (swim) were significantly elevated ($p<0.05$) above Groups 1 (handle) and 2 (restraint) on days 4, 11, 18 and 32, but they were not statistically different from each other. Likewise, Groups 1 and 2 did not differ significantly. The elevated plasma corticosterone peaks on the 5th day of weekly stress continued over the 6-week exposure for Groups 3 and 4. In order to approximate the levels of corticosterone before and after stress on the first 4 days of the week, animals sacrificed on day 43 (Tuesday) were divided into 2 subgroups. In each treatment group, 4/8 animals were stressed prior to sacrifice and the remaining 4/8 animals were not so stressed. The results of the corticosterone assays for these animals are shown in Figure 2.

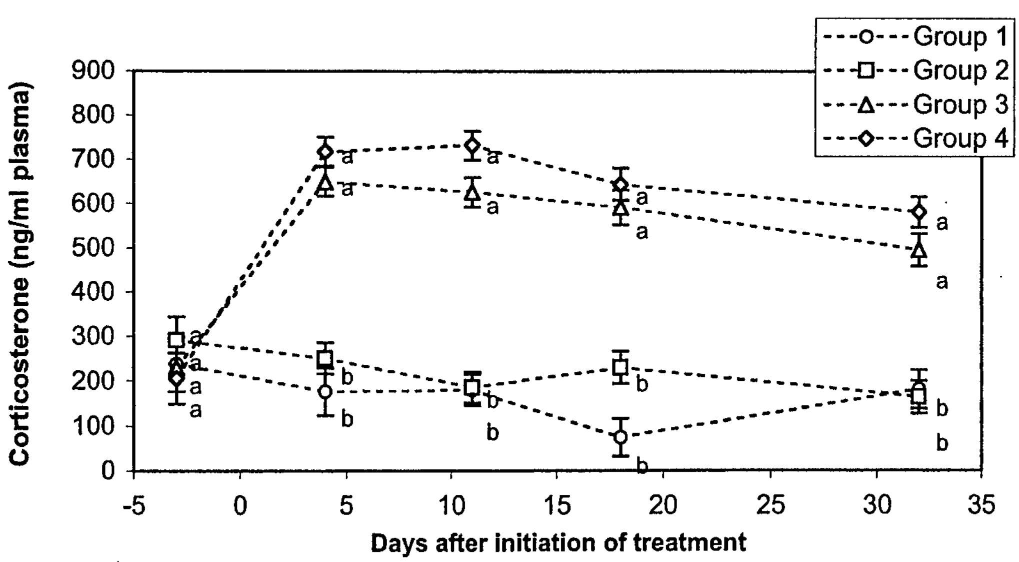


Figure 1. Corticosterone levels of animals by treatment group and study day. Blood for plasma corticosterone analysis was collected after the cessation of the fifth day of weekly stress. Each point represents the mean of 8 observations (± standard error of the mean). Means within a day with no letters in common are significantly different at α=0.05 according to Bonferroni-corrected multiple comparison.

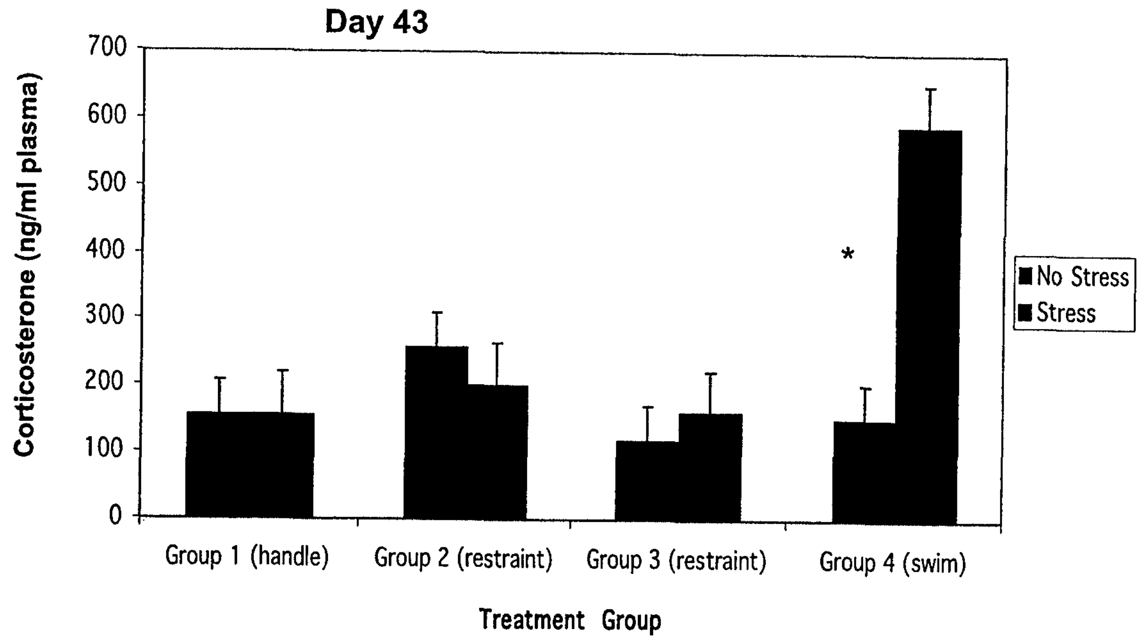


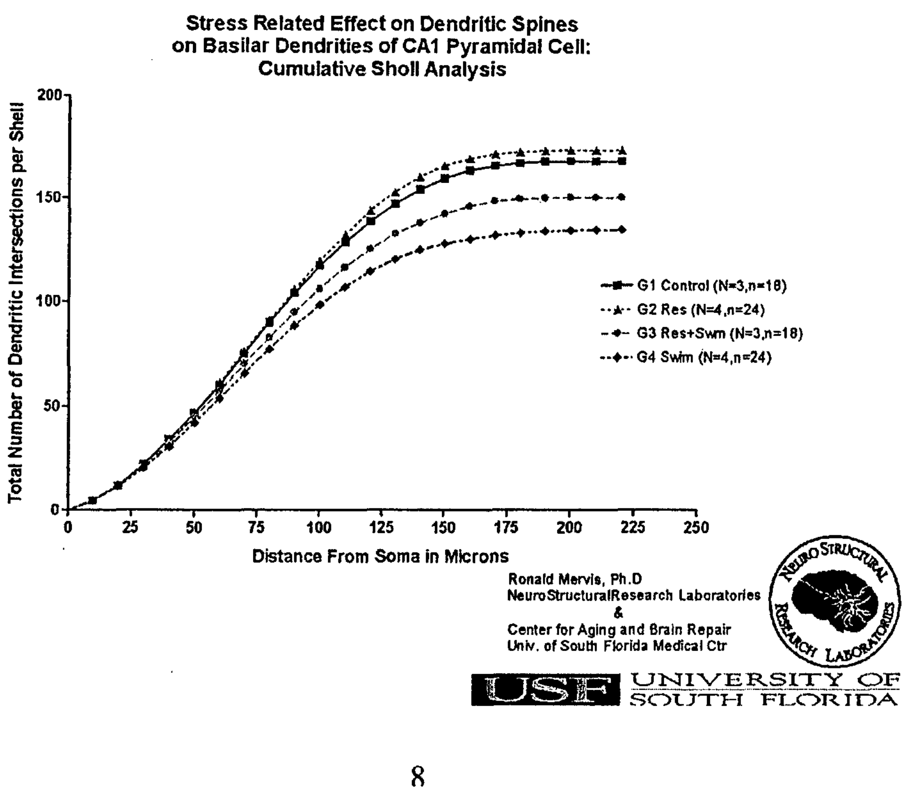
Figure 2. Corticosterone levels of animals by treatment group on study day 43 (2nd day of weekly stress). Stress/no stress indicates presence or absence of presacrifice stress episodes in each group. Blood for plasma corticosterone analysis was collected after the cessation of stress. Each point represents the mean of 4 observations (± standard error of the mean). * indicates a significant difference between no stress and stress in that treatment group at $\alpha=0.05$ according to test of simple main effect.

These findings indicate that elevation of plasma corticosterone seen in Groups 3 and 4 (Figure 1) is directly associated with swimming stress, and that such elevations are transient. As an example, Group 4 rats that were swum on day 43 had elevation of plasma corticosterone (Figure 2, Group 4, stress). Other members of that group, who had last swum on day 42 (the day before blood samples were obtained, Table 1) had normal levels of this hormone on day 43 (Figure 2, Group 4, no stress). It thus appears that post-swimming elevation of this hormone returned to normal within 24 hours. Similarly, Group 3 rats, last swum on day 39, had normal plasma corticosterone levels on day 43, whether or not preterminal restraint was applied (Figure 2). As also reported in the October 2003 Annual Report, stress also affected body weight beginning on day 18. Body weights of animals in Groups 2, 3 and 4 were consistently lower than control Group 1, but only Group 4 animals were significantly different ($p<0.05$) from Group 1.

An ancillary investigation was conducted at the end of the study with these animals, to see if these stress models would affect acute (cholinergic) toxicity of the organophosphate chlorpyrifos. The latter was administered on day 39 (after data for Figure 1 was collected) to 4/8 animals/group. Sacrifice was on day 43. This was presented at Bioscience Review 2004, Hunt Valley, MD, and is in press in the Proceedings of that meeting (see manuscript of Hancock *et al.*, Reportable Outcomes and Appendix, below). In summary, there was no stress-related alteration of brain chlorpyrifos-induced acetylcholinesterase inhibition. In addition, there was no chlorpyrifos associated alteration of plasma corticosterone. Data from this study were presented at the November 2003 meeting of the Society for Neuroscience (see Hancock *et al.* abstract in Reportable Outcomes, below).

In work done in the year currently being reported, cerebral hemispheres from four rats of each group were sent to Dr. Ronald Mervis, NeuroStructural Research Laboratories, University of South Florida, Tampa, FL, who performed Golgi staining and related quantitative analysis (including Sholl analyses) of dendrites in hippocampal pyramidal cells (Flood; Morest). There was atrophy of basilar dendrites in the CA1 neurons, using criteria such as dendritic length and basilar branching. These changes were seen in the swim (Group 3) and restraint plus swim (Group 4) rats compared to handling control (Group 1) and restraint (Group 2) animals (Figure 3). The groups paired by the data (1 and 2, 3 and 4) did not differ from one another. As an example, the measures of the estimated length of the dendrites of the CA1 tree indicated the following (as percent of control): control- 100%, restraint- 103%, restraint plus swim- 89%, swim- 80% (one way ANOVA p= 0.144, Student Newman Keuls posthoc test- control greater than swim- $p< 0.05$, restraint greater than swim- $p< 0.05$). These data indicate that swimming caused the greatest dendritic atrophy, and that restraint plus swim did not cause more of this change than swim alone.

Figure 3. Cumulative Sholl analysis of basilar dendrites of Golgi stained CA_1 pyramidal neurons.



D. ***Task 4***- Perform an **acute toxicity study** evaluating the effects of several doses of soluble DU and of stress, as measured by neurobehavioral, neurochemical and neuropathological and regional brain uranium concentrations. The in-life part of the study and much of the data analyses were performed in Year 3. This study followed the format recommended at the December 2002 Force Health Protection Review of DU projects, reflected in the Statement of Work.

1- *Study Design-*

a- DU dosing- The approach featured the use of single intramuscular doses of 0.1, 0.3 and 1.0 mg/kg of soluble DU (uranyl acetate) in male Sprague-Dawley rats. There was one week predosing of administration of stress consisting of four consecutive days of restraint stress followed by one day of forced swimming. At the cessation of stress, animals were bled for subsequent corticosterone determination and immediately dosed intramuscularly with DU. This resulted in transient elevation of plasma corticosterone at the time of dosing (see below). The study design is provided in Table 2.

b- Neurobehavioral in-life assessments- The Functional Observational Battery, motor activity assessment and passive-avoidance testing were performed weekly (see Table 3, see Appendix for details of tests).

c- Assessments at sacrifice (days 1, 3, 7 and 30)- Rats were sacrificed on the indicated days, with the following determinations being done. One cohort (n=5/dose/day) were used for neurochemical evaluation of regional brain neurotransmitters, their receptor levels and the intensity of oxidative stress (see Appendix, Methods, for list of neurochemical assays), renal pathology and clinical biochemistry. A second similar sized cohort was used for ICP-MS analysis of blood and regional brain uranium levels (samples from the 0.1 mg/kg group were not collected for ICP-MS analysis after post-dosing day 1). A third cohort was perfusion-fixed for detailed neuropathology (n=5/group/day, not done on sacrifice day 1). Thus the overall n/group/day was up to 15, and total number in the study was 418 rats. The experimental design has four treatments (three dose levels of DU plus control), four sacrifice intervals (days 1, 3, 7, 30), and two stress states (yes or no) (Table 2). This number of rats and workload (such as daily stress) necessitated performing the in-life portion of the study in five experimental periods (blocks), termed DUA 1 through 5.

Table 2. Treatment design for each experimental block of the acute exposure study.

Group	Stress	DU dose	Total Animals	n/group	Day 1	Day 3	Day 7	Day 30
1	**no**	**0**	pathology*=3, neurochem*=4, icp-ms*=4	**11**	pathology =0 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1
2		**0.1**	pathology=3, neurochem=4, icp-ms=1	**8**	pathology =0 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=0	pathology =1 neurochem=1 icp-ms=0	pathology =1 neurochem=1 icp-ms=0
3		**0.3**	pathology=3, neurochem=4, icp-ms=4	**11**	pathology =0 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1
4		**1**	pathology=3, neurochem=4, icp-ms=4	**11**	pathology =0 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1
5	**yes**	**0**	pathology=3, neurochem=4, icp-ms=4	**11**	pathology =0 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1
6		**0.1**	pathology=3, neurochem=4, icp-ms=1	**8**	pathology =0 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=0	pathology =1 neurochem=1 icp-ms=0	pathology =1 neurochem=1 icp-ms=0
7		**0.3**	pathology=3, neurochem=4, icp-ms=4	**11**	pathology =0 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1
8		**1**	pathology=3, neurochem=4, icp-ms=4	**11**	pathology =0 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1

*pathology = animals used for neuropathological and kidney lesion evaluation
neurochem = animals used for neurochemical evaluation of regional brain transmitters, receptor levels and oxidative stress, kidney pathology
icp-ms = animals used for inductively coupled plasma-mass spectrometry analysis of uranium concentration (no samples collected for low dose group after post-dosing day 1)

Below is the study schedule for each experimental block:

Table 3. Schedule of activities for each experimental block of the acute exposure study.

SUN	MON	TUES	WED	THURS	FRI	SAT
Day -12	**Day -11**	**Day -10**	**Day -9**	**Day -8**	**Day -7**	**Day -6**
		animals out of quarantine			rotarod training	
Day -5	**Day -4**	**Day -3**	**Day -2**	**Day -1**	**Day 0**	**Day 1**
	rotarod training passive avoidance (PA) training	rotarod training PA retention/retrain		Functional Observational Battery (FOB) Motor Activity (MA)	restraint stress anesthesia chamber adaptation	restraint stress anesthesia chamber adaptation
Day 2	**Day 3**	**Day 4 (Dosing day)**	**Day 5**	**Day 6**	**Day 7**	**Day 8**
restraint stress anesthesia chamber adaptation	restraint stress anesthesia chamber adaptation	swim stress (a.m.) blood collection (for CORT assay) and **DU injection after stress**	**Sacrifice** -24 hour post-dosing		**Sacrifice** 3 days post-dosing	
Day 9	**Day 10**	**Day 11**	**Day 12**	**Day 13**	**Day 14**	**Day 15**
	FOB/MA	**Sacrifice** 7 days post-dosing	PA retention			
Day 16	**Day 17**	**Day 18**	**Day 19**	**Day 20**	**Day 21**	**Day 22**
	FOB/MA		PA retention			
Day 23	**Day 24**	**Day 25**	**Day 26**	**Day 27**	**Day 28**	**Day 29**
	FOB/MA		PA retention			
Day 30	**Day 31**	**Day 32**	**Day 33**	**Day 34**		
	FOB/MA		PA retention	**Sacrifice** 30 Day post-dosing		

2- *Results of the Acute Study*

a- Corticosterone Levels at Dosing- The plasma corticosterone levels of unstressed (Groups 1-4, Table 2) and stressed (Groups 5-8, Table 2) animals at the time of DU dosing were 189.8 +/- 90.81, n=200 and 763.65+/- 130.94, n= 206 (ng/ml, mean +/- Standard Deviation) respectively. This is clearly related to the week of restraint/swim stress. From the pilot stress study data (above), this elevation is most likely related to the swimming episode, which was done shortly before the DU dosing.

b- Neurobehavioral Findings- These consisted of almost weekly use of the Functional Observational Battery, motor activity evaluations and the passive avoidance test of learning and memory. Data from these determinations are given in Tables A1, A2, A3, A4 in the Appendix. The major findings were as follows. Treatment with uranium significantly ($P=0.002$) decreased forelimb grip strength across all levels of uranium treatment in unstressed rats. Grip strength decreased from 1.95 ± 0.02 kg (N=101) in control animals to 1.84 ± 0.03 kg (N=101) in uranium treated animals. Prior exposure to stress mitigated this effect of uranium on grip strength (Table A2). Treatment with DU decreased the rate of weight gain ($P<0.0001$) in treated rats (Table A2, Figure 4). The effect was most pronounced on day10 (the first post-dosing day measured), when average weight of controls was 340 g. and uranium treated rats was 318 g. This gap closed during the remainder of the 30 day study (Figure 4). Prior exposure to stress did not alter the effect of uranium exposure on weight gain. Exposure to uranium also produced dose dependent decreases in ambulatory activity ($P=0.0078$), (Table A3, Figure 5). Effects were most pronounced on day 10 but remained significant at all times. Prior exposure to stress did not alter this effect of uranium on ambulatory activity. There were modest, scattered DU treated-associated changes in arousal, response to click or pinch, and temperature, but these were not severe and/or temporarily consistent enough to be considered of biological significance (Tables A1, A2).

Figure 4. Body weight of control and uranium treated rats.

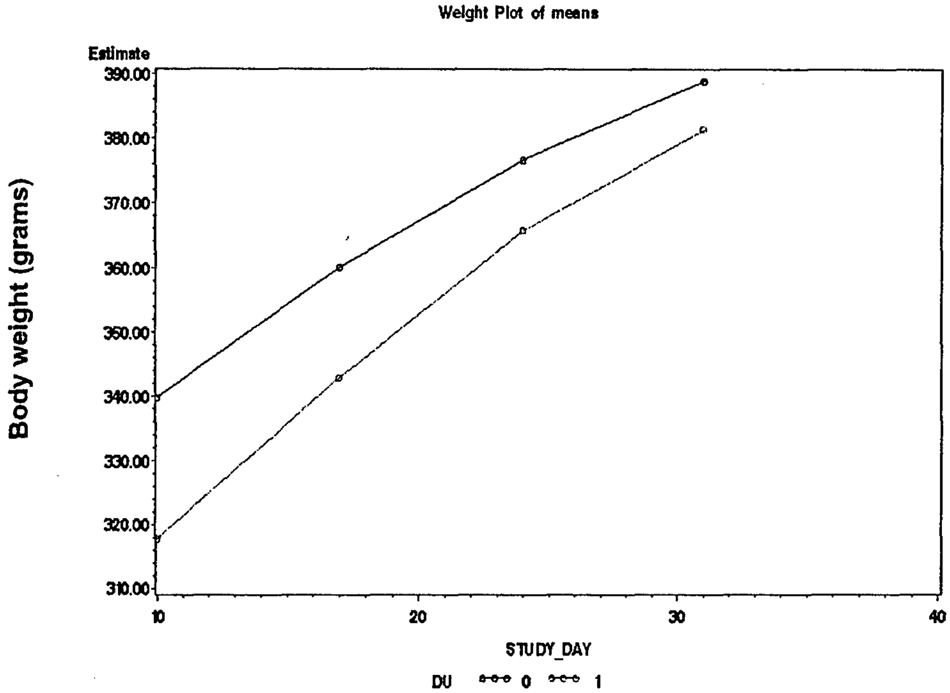
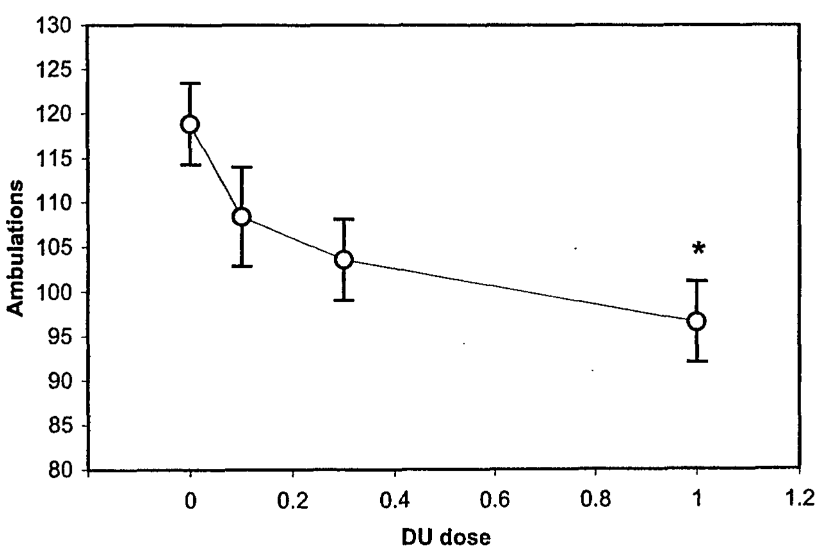


Figure 5. Ambulatory beam breaks across all days by DU dose. Note: Each point represents the mean of 80-119 observations, error bars indicate ± standard error of the mean (based on the pooled mean square error).



c- Tissue Uranium- Uranium levels in brain cortex, cerebellum, hippocampus, striatum, serum, and kidney were determined by inductively coupled plasma- mass spectrometry (ICP-MS) following acid digestion. The analysis was performed by the Analysis Section of the University of Arizona Southwest Hazardous Waste Program Hazard Identification Core. We have analyzed approximately 50% of the brain and serum samples (from blocks DUA 1, 2 and part of 3). In these samples, tissue and serum levels appear greatest 3 days after administration. Dose dependent increases were observed in brain and serum uranium levels, though at the 0.1 mg/kg dose, changes in the brain uranium levels may be insignificant. Peak uranium levels occurred later than observed in our previous study (see Reportable Outcomes and Appendix- Barber *et al.*, in press) and likely reflects slower absorption of uranium from intramuscular injection than intraperitoneal injection. No kidney samples have as yet been assayed.

d- Neurochemistry
(I)- Neurotransmitter Content and Receptor Numbers. Tissue concentrations of gamma-amino butyric acid (GABA) were determined in cortex, cerebellum, striatum, and hippocampus by HPLC following o-phthalaldehdye derivatization (Peinado, *et al.*) (Appendix - Table A20). No significant treatment group by time after administration interactions were observed in any brain region, indicating that neither uranium nor prior stress treatment have a significant effect on brain regional GABA content. Dopamine, serotonin, and norepinephrine were determined by reversed phase HPLC with electrochemical detection in cortex, cerebellum, caudate, and hippocampus (Appendix - Tables A5-A19). Rats treated with 1 mg/kg uranyl acetate exhibited a 40% decrease in caudate putamen dopamine content 3 days after dosing (Table A18). This effect on dopamine was ameliorated by prior application of stress (Table A18). No effect of DU or stress was observed on levels of GABA, serotonin, norepinephrine (Tables A20, A5-A9, A10-A14). Glutamate analysis has not been completed at this time.

(II)- Receptors. Receptor numbers are being determined for dopamine D2 receptors in striatum, nACh receptors in cortex, and NMDA receptors in hippocampus by saturation radioligand binding. These experiments are still in progress.

(III)- Glutathione- The amounts of reduced (GSH) and oxidized (GSSG) glutathione are commonly used measures of cellular redox state. Decreases in GSH or increases in GSSG are indicative of a more oxidizing environment within the cell. GSH and GSSG were measured in cortex, cerebellum, caudate-putamen, and hippocampus by HPLC following derivitization with fluorodinitrobenzene (Fariss *et al.*) (Appendix - Table A21). At this point (assays have been completed for 3/5 of the animals in each group/ sacrifice day), no significant effect of treatment has been observed, though there were regionally different levels with hippocampus and caudate being higher than cortex and cerebellum (Table A21).

e- Neuropathology- Light microscopic examination has been made of the brains and eyes. These tissues were collected from the perfusion-fixed rats (n=5/doseage level/interval). The brain was trimmed into five transverse planes demonstrating most of the major brain structures (n=4/5 rats per group/day), and all the eyes in a single mid-saggital one. The tissues were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined by light microscopy (by veterinary neuropathologist B. Jortner). No lesions were detected. Immunohistochemical stains for glial fibrillary acidic and Fluoro-Jade stains for neuronal degeneration (Schmeud and Hopkins) have not been evaluated. Sections of spinal cord and peripheral nervous system from perfusion-fixed rats that were post-fixed and embedded in epoxy resin have been in part sectioned, but not examined.

f- Renal pathology- The kidneys were collected from rats that had been part of the sacrifices for neurochemistry, and thus were a different cohort from those evaluated for neuropathology. These animals (n= 5/dose/interval) also had blood taken at sacrifice for clinical chemistry. The kidneys were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined by light microscopy. Renal lesions included prominent acute having a DU (uranyl acetate) dose-related severity. This was even noted one day after DU exposure. These changes were primarily noted in the inner cortex and outer medullary stripe of the kidney, with extension along the medullary rays in mid and high dose treatment groups. Tubular regeneration occurred rapidly in low dose (0.1 mg/kg) animals and was almost complete by day 30. High dose animals had more extensive tubular necrosis and a relatively delayed regenerative response, with regions of multifocal chronic interstitial nephritis and cortical scarring on day 30. Mid dose (0.3 mg/kg) treatment animals displayed lesions of intermediate severity. These changes were reflected in the clinical chemistry determinations (Table A22). There were dose-related elevations of serum blood urea nitrogen and creatinine, which were evident at day one (for creatinine), peaked by day three and had largely returned to control levels by day 30. Associated decline in serum albumin and hematocrit was also noted. Stress had no effect on the nephrotoxicity. These findings have been submitted for presentation at the 2005 Society of Toxicology meeting (see abstract- Tobias *et al.* In Reportable Outcomes and Appendix)

3- *Summary and Interpretation- Acute Study-*
The results of this study demonstrate that a single exposure to soluble DU (uranyl acetate) at doses as low as 0.1mg/kg can have adverse neurological effects. This includes toxin-associated reduction in dopamine levels in the caudate-putamen, and alteration in some neurobehavioral activities. In no case were these effects exacerbated by prior stressful stimuli and in at least one instance (dopamine decrease), the effects were prevented by prior exposure to stress. Changes in caudate-putamen dopamine were observed on day 3, however, they only occurred in the highest dose (1.0 mg/kg) and were transient, returning to control values by day 7. It is possible that receptor number analysis (in progress) will reveal subtle changes that account for the observed neurotransmitter effect. There was no observed associated histopathological change in this region, when studied using routine (hematoxylin and eosin) tissue staining, but immunohistochemistry for tyrosine hydroxylase would be useful in exploring this alteration. Of interest were the DU exposure-related clinical effects, including diminished grip strength, motor activity and body weight gain. It is possible that these may be secondary to the DU-induced kidney injury and transient uremia, which was observed at all uranium doses (in a dose-related fashion). However, prior stress had little effect on uranium renal toxicity, but appeared to alter some of the neurological effects. It needs to be noted that not all samples from this study have been analyzed for all variables. This particularly includes assays for reduced and oxidized glutathione and tissue uranium concentration, along with pathological examination of the peripheral nervous system and adrenal gland.

E. ***Task 5-*** Perform a **long-term (chronic) toxicity study** evaluating the effects of several doses of implanted DU pellets and continuous stress, as measured by neurobehavioral, neurochemical and neuropathological and regional brain uranium concentrations. This task was modified from the original Statement of Work, based upon review of the initial year's results at the December 2002 Force Health Protection Review. Specifically, the study was changed to provide periodic peaks of elevated plasma corticosterone during the entire six-month study by inducing stress 5 days/week with a routine stressor (restraint) and a superimposed periodic novel stress (swimming) as a model of militarily relevant stress. The results of the pilot study performed for Task 3 showed that the stress model tested, which consisted of a weekly regimen of 4 consecutive days of restraint followed by one day of swimming, produced a 3 to 4-fold increase in plasma corticosterone on the swim day only. This trend was observed for the entire 6 weeks tested. However, the results also showed that the animals habituated to the restraint quite quickly and there was no significant difference between the plasma corticosterone levels of animals stressed by restraint compared to the controls. As a result, we have decided to use a variable, unexpected stress model for the chronic study. This model uses a weekly random pattern of 3 days of restraint, 1 day of swimming and 1 day of handling, with the anticipation that the variable pattern might produce plasma corticosterone peaks from stress treatments other than swimming. The study is designed so animals in each experiment are stressed in the same pattern for the same week of the study. (i.e. during Week 2 of each experiment, animals will be swum on Monday, restrained on Tuesday, handled on Wednesday, restrained on Thursday and Friday; during Week 3, animals will be handled on Monday, restrained on Tuesday and Wednesday, swum on Thursday and restrained on Friday; during Week 4, animals will be restrained on Monday, swum on Tuesday, restrained on Wednesday, handled on Thursday and restrained on Friday, etc.). See Table 5 for the weekly stress pattern for the entire study.

A description of the chronic study, which is currently in progress follows.

1- *Experimental Design-*
   The study is designed with a 2X4 factorial array treatment structure (2 levels of stress, 4 levels of depleted uranium [including negative control]). Five (overlapping) replicate experiments are being conducted with terminal sacrifices at 6 months and three terminal endpoints, as in the acute study (neurochemistry, icp-ms determination of tissue uranium concentration, pathology). For each experiment, there will be 8 treatment groups, with 3 animals/treatment group to give n=1 animal/treatment group/terminal endpoint/experiment and an n=5 animals/treatment group/terminal endpoint for the study (n=24 animals/experiment). Dosing for treatment groups consists of implantation of tantalum pellets (control), DU pellets or a combination in the gastrocnemius muscles of each rat (10 pellets/leg). The treatment structure for each experiment is as follows (Table 4):

Table 4. Treatment design for each experimental block of the chronic exposure study.

Trt Group	stress	UA dose	Use	No. Ta pellets/leg	No. DU pellets/leg	Total Ta. pellets/rat	Total DU pellets/rat	*N*
1	0	0	icp-ms	10	0	20	0	1
1	0	0	nchem	10	0	20	0	1
1	0	0	path	10	0	20	0	1
2	0	low	icp-ms	8	2	16	4	1
2	0	low	nchem	8	2	16	4	1
2	0	low	path	8	2	16	4	1
3	0	mid	icp-ms	5	5	10	10	1
3	0	mid	nchem	5	5	10	10	1
3	0	mid	path	5	5	10	10	1
4	0	high	icp-ms	0	10	0	20	1
4	0	high	nchem	0	10	0	20	1
4	0	high	path	0	10	0	20	1
5	1	0	icp-ms	10	0	20	0	1
5	1	0	nchem	10	0	20	0	1
5	1	0	path	10	0	20	0	1
6	1	low	icp-ms	8	2	16	4	1
6	1	low	nchem	8	2	16	4	1
6	1	low	path	8	2	16	4	1
7	1	mid	icp-ms	5	5	10	10	1
7	1	mid	nchem	5	5	10	10	1
7	1	mid	path	5	5	10	10	1
8	1	high	icp-ms	0	10	0	20	1
8	1	high	nchem	0	10	0	20	1
8	1	high	path	0	10	0	20	1

*pathology = animals used for neuropathological and kidney lesion evaluation
neurochem = animals used for neurochemical evaluation of regional brain transmitters, receptor levels and oxidative stress
icp-ms = animals used for inductively coupled plasma-mass spectrometry analysis of uranium concentration

2- *Study Activities/Calendar* (Day/Week 0 is the first day/week that DU pellets are surgically implanted):

a- Quarantine (Week-1): Upon receipt, animals are quarantined for 7 days and are housed singly.

b- Pellet implants (Week 0-2): Six to nine days after animals are removed from quarantine, tantalum, DU pellets or a combination are implanted in the gastrocnemius muscle of each rat (10 pellets/leg). The pellets are implanted using sterile surgery technique (see Methods in Appendix for details of pellet implant procedure). The animals are allowed to recover for approximately 2 weeks.

c- Stress (Weeks 2-24): Stress begins 12-14 days after the pellets are implanted and continues 5 days/week for 22 weeks. Table 5 shows the stress patterns for each week of the study.

Table 5. Weekly stress pattern for variable stress design of chronic exposure study.

Study Week	Day of the Week				
	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY
2	swim	restraint	handle	restraint	restraint
3	handle	restraint	restraint	swim	restraint
4	restraint	swim	restraint	handle	restraint
5	swim	restraint	restraint	handle	restraint
6	restraint	swim	handle	restraint	restraint
7	restraint	handle	restraint	swim	restraint
8	restraint	restraint	handle	restraint	swim
9	restraint	restraint	restraint	handle	swim
10	swim	restraint	restraint	restraint	handle
11	restraint	restraint	restraint	swim	handle
12	handle	swim	restraint	restraint	restraint
13	restraint	restraint	handle	swim	restraint
14	restraint	handle	swim	restraint	restraint
15	swim	handle	restraint	restraint	restraint
16	restraint	swim	restraint	restraint	handle
17	restraint	restraint	swim	restraint	handle
18	handle	restraint	restraint	restraint	swim
19	handle	restraint	swim	restraint	restraint
20	restraint	restraint	swim	handle	restraint
21	restraint	handle	restraint	restraint	swim
22	restraint	handle	swim	restraint	restraint
23	swim	restraint	restraint	handle	restraint
24	swim	handle	restraint	restraint	restraint

Denotes bleeding days

d- Blood collection (Weeks 2, 6, 11, 14, 17, 22): To monitor the effects of stress treatment, blood is collected from the orbital sinus every 4 weeks to determine plasma corticosterone levels. Four samples/stress treatment (restraint, swim, handle)/time point, including nonstressed animals, will be collected for each experiment to give an n=20 for each time point and stress treatment for the entire study. Samples are collected on three days of the week to obtain measurements of plasma corticosterone in nonstressed animals (handle only) and animals exposed to the three different stressors (handling, restraint and swimming). See Table 5 for bleeding schedule. Blood is collected immediately following the stress session.

e- Neurobehavioral Studies-

(I) Motor Activity measurements are collected at Week –1 (before pellets are implanted), and in Weeks 1, 4, 8, 11, 14, 17, 20, 23..

(II) Functional Observational Battery (FOB) will be performed at Week –1, and in Weeks 1, 4, 8, 11, 14, 17, 20, 23.

(III) Learning/Memory Tests: Tests of passive and active avoidance will be performed in the last 2 months of the study.

f. Terminal Sacrifice will be as in the acute studies, with the addition of urine collection for uranium concentration determination. Briefly, this includes neurochemical evaluation (n= 5/group) of regional brain neurotransmitters (dopamine, serotonin, norepinephrine, glutamate and GABA for cerebellum, cortex, hippocampus and caudate putamen), their receptor levels and the intensity of oxidative stress (reduced and oxidized glutathione determinations), ICP-MS analysis of blood and regional brain uranium levels (n= 5/group); detailed neuropathology (n=5/group), renal pathology (n=5/group) and clinical biochemistry (n=5/group).

F. Activities Outside Statement of Work- Two major activities outside of the Statement of Work occurred. In response to the request at the review of our project at the Force Health Protection Review of Depleted Uranium Projects in December 2002 for enhanced collaboration among investigators involved in the several funded DU projects, we are working with Dr. Michael Aschner, Vanderbilt University Medical Center. This involves assays of divalent metal transporter-1 (DMT-1) levels, a measure of potential uranium transport from blood to brain. We have supplied Dr. Aschner with frozen (- 70° C) samples from the cerebral cortex, caudate-putamen, hippocampus and cerebellum from rats sacrificed for neurochemistry in the Acute Study (see Task 4, above). The DMT-1 assays will be performed in his laboratory. These studies make use of experimental material already available, to provide information concerning mechanism of uranium transport into the brain.

The second activity involved sending technical staff (Sandra Hancock and Jonathan Hinckley) and our medicine laboratory animal veterinary resident (Dr. Amy Tanner) to the Armed Forces Radiobiology Research Institute (AFRRI) Bethesda, MD to obtain training in the surgical implantation of DU pellets in rats. This was arranged and organized by Dr. David McClain of AFRRI, who also was very helpful in directing us to commercial sources of for DU and tantalum pellets for use in the chronic (implantation) study.

II. Problems Encountered, Solutions and Recommended Approaches to Future Work. The major problem encountered was the time it would take to complete aspects of this large, complex study, and my lack of appreciation of this fact when formulating the schedule. When the project was expanded, in early 2003, following the December 2002 Force Health Protection Review of DU projects, I anticipated the project could be completed with the addition of one year, which would end it September 30, 2005. Experience has shown that this was an overly optimistic assessment. The in-life portions of the project were slightly delayed, to allow for renovations to our laboratory animal facility, and more significantly to allow for training of our staff in the surgical procedures involved in the long-term (DU implantation) study. As the name implies, this final part of the project requires a six-month exposure to the implanted DU, and was initiated on September 6, 2004. Given the complexity (periodic administration of stress, neurobehavioral assessments and blood collection) of the in-life portion of the study, it will need to be done in five overlapping 25 week blocks, with an ending date of June 20, 2005. Experience in the acute study has shown us how long the post-sacrifice tissue analyses take. This is particularly true of the high volume of icp-ms uranium analyses we need, for which tissue preparation is done at the University of Florida and the assay at the Analysis Section of the University of Arizona Southwest Hazardous Waste Program Hazard Identification Core. Since the workload at the latter institution is great, there often is a delay in analyzing our samples. Another part that has been slower than I originally anticipated is the preparation and evaluation of sections from epoxy resin embedded peripheral nervous system, which is done in my laboratory at Virginia Tech. Technicians who do this work are also involved in in-life aspects of the study, and hence the time they can devote to such sectioning is limited when animals are on the study. Given the proceeding, and the extent of analysis of other materials collected at sacrifice, we clearly will not be able to complete that work by the now approved September 30, 2005 ending date. This, we will need to ask for a one-year extension of the project. This will need to be reflected in a modification of the Statement of Work.

A second problem in the just completed acute study has been the presence of renal tubular degeneration relating to even the lowest (a single dose of 0.1 mg/kg) exposure to soluble DU (uranyl acetate). Thus, we need to evaluate neurotoxic changes associated with exposure to soluble DU in the face of the renal lesions. This should not be a problem in the long-term (DU implantation) study now in progress.

Key Research Accomplishments- Year Three

- Two paper in press (see Reportable Outcomes and Appendix).

  - Barber, D.S., Ehrich, M. Jortner, B.S. Distribution and kinetics of uranium in the rat brain after intraperitoneal injection of uranyl acetate. Journal of Toxicology and Environmental Health.

  - Tolson K, Pomeroy M, Roberts S, Barber D. Role of heat shock proteins in resistance to uranium nephrotoxicity. Toxicology.

- Presentation of findings from the pilot stress study.

  - S.K. Hancock, M.F. Ehrich, J. Hinckley, T. Pung, K.L. Farris, B.S. Jortner. Stress and neurotoxicant exposure. Comparison of effects of several stress models on the acute neurotoxicity of the organophosphate chlorpyrifos (see Appendix). 2003 Society for Neuroscience meeting, New Orleans, LA.

  - S.K. Hancock, M.F. Ehrich, J. Hinckley, T. Pung, B.S. Jortner. The effect of stress on the acute neurotoxicity of the organophosphate chlorpyrifos. Bioscience Review 2004, Hunt Valley, MD. Note- This is in press in Proceedings of this meeting (see Reportable Outcomes and Appendix).

- Completion of evaluation of Golgi staining of brains from pilot stress study, indicating that multiple exposure to swimming stress led to dendritic atrophy in CA1 hippocampal neurons (from Dr. Ron Mervis).

- Completion of most of the acute toxicity study using soluble DU, as noted in this Report.

- Two papers submitted for presentation at the 2005 meeting of the Society of Toxicology (see Reportable Outcomes and Appendix).

  -Barber, DS, Hancock, SK, McNally, AM, Hinckley, J, Binder, E, Ehrich, MF and Jortner, BS. Neurological effects of acute uranium exposure.

  -L. Tobias, D. Barber, K. Zimmerman, S. Hancock, J. Hinckley, M. Ehrich, B, S. Jortner. Nephrotoxic effects of depleted uranium (DU) in the rat. Interactions with stress.

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- Initiation of the long-term (DU implantation) study. This includes acquisition of surgical skills by laboratory personnel, so that the DU pellet implantation could be carried out.

Reportable Outcomes

One paper was presented at the 2003 annual meeting of the Society for Neuroscience-

S.K. Hancock, M.F. Ehrich, J. Hinckley, T. Pung, K.L. Farris, B.S. Jortner. Stress and neurotoxicant exposure. Comparison of effects of several stress models on the acute neurotoxicity of the organophosphate chlorpyrifos (see Appendix).

Two abstracts have been accepted for presentation at the 2005 meeting of the Society of Toxicology

NEPHROTOXIC EFFECTS OF DEPLETED URANIUM (DU) IN THE RAT. INTERACTIONS WITH STRESS. L. Tobias, D. Barber, K. Zimmerman, S. Hancock, J. Hinckley, M. Ehrich, B. S. Jortner, Virginia Tech, Blacksburg, VA and University of Florida, Gainesville, FL

NEUROLOGICAL EFFECTS OF ACUTE URANIUM EXPOSURE. Barber, DS[1], Hancock, SK[2], McNally, AM[1], Hinckley, J[2], Binder, E[2], Ehrich, MF[2] and Jortner, BS[2]. [1] Center for Environmental and Human Toxicology, University of Florida, Gainesville, FL, USA [2] Laboratory for Neurotoxicity Studies, VPI&SU, Blacksburg, VA, USA

One paper was presented at the Bioscience Review, May 2004 (Manuscript is in press).

The Effect of Stress on the Acute Neurotoxicity of the Organophosphate Chlorpyrifos., S. Hancock, M.F. Ehrich, J. Hinckley, T. Pung, B.S. Jortner. Laboratory for Neurotoxicity Studies, College of Veterinary Medicine, Virginia Tech, Blacksburg, VA, USA

Two papers from earlier parts of the project are in press.

Barber, D.S., Ehrich, M. Jortner, B.S. Distribution and kinetics of uranium in the rat brain after intraperitoneal injection of uranyl acetate. Journal of Toxicology and Environmental Health.

Tolson JK, Roberts S, Pomeroy M, Jortner B., Barber D. Role of heat shock proteins in resistance to uranium nephrotoxicity. Toxicology.

Conclusions

Major conclusions drawn from work completed in this year (Year 3 of the project) are as follows.

Pilot Stress study- The data from the Golgi staining of hippocampal neurons (done by Dr. Ron Mervis, University of South Florida) were assessed this year. These results indicate that swimming stress, in addition to eliciting transient elevations of plasma corticosterone, when applied multiple times over a six-week period results in dendritic atrophy in basilar regions of the dendrites of the CA1 region.

Acute Study (using soluble DU)- There appears to be DU dose-associated, transient reduction in dopamine content of the caudate-putamen. This was seen on post-dosing day 3, only at the highest (1.0 mg/kg DU) dose of this metal, with protection offered by prior exposure to stress. Values returned to normal when studied on day 7. Several clinical changes were noted in animals on the study, most prominently DU-associated reduced forelimb grip strength, ambulatory movement and body weight gain. These were most marked in the post-dosing period, with some recovery noted later in the study. Prior stress offered protection only in the case of grip strength. These changes are paralleled by the DU-dose-related acute tubular necrosis in the kidneys, a feature unaffected by stress. The renal tubular epithelium regenerated later in the study. Thus, we give strong consideration to a role for the renal disease in the evolution of these clinical effects. Prior stress did not enhance any toxic effects.

Recommendations for Future Work- These findings suggest that DU exposure impacts upon an important neurotransmitter system. This needs to be confirmed, and work using immunohistochemistry for dopaminergic neurons and their processes, using primary antibodies to tyrosine hydroxylase in sections from the caudate putamen and substantia nigra. This would not require additional animal dosing, as it can be done in material already in hand. In addition, more direct studies could be made using direct application of soluble DU to organ slices/cultures *in vitro*. Given the presence of dendritic atrophy in hippocampal neurons in our stress model (which is not unexpected), and the importance of that brain region in learning and memory, there appears to be a need to see if long-term exposure to implanted DU would exacerbate this effect. This would have military relevance, given the potential for long-term stress and DU exposure in such environments. Again, this would not involve additional animal experiments, as appropriate material can be obtained from current studies.

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Appendix

Statement of Work-

Methods-

Raw Means and P-Value Tables-

Abstracts-

Manuscripts-

STATEMENT OF WORK- Revised 1/10/03

The proposed work will use the male Sprague Dawley rat to characterize the kinetics and toxicity of depleted uranium (DU) in the brain. The ability of stress to affect disposition and toxicity of DU will also be examined, as stress can alter the permeability of the blood-brain barrier and enhance neurodegeneration. The studies would examine neurotoxicity of DU with and without stress in acute and long-term exposures. Assessment would include detailed morphological, neurobehavioral, neurochemical and toxicokinetic methods.

The experimental design will be a split plot design. The whole plot will be a factorial array in a randomized complete block design. The treatments are stress (2 levels- stressed and unstressed) and DU dose (2-4 levels including negative controls). The whole-plot is rat and the sub-plot is brain region (4 regions). For the **kinetic study**, a 2x2 design will be used (2 doses of DU and 2 levels of stress [stressed or unstressed]). The stressor will be applied once daily for at least 5 days prior to DU exposure. Uranium levels in cortex, hippocampus, caudate-putamen, and cerebellum, as well as in blood, will be determined at several times after DU exposure (e.g., 8 hours, 1 day, 7 days, and 30 days). For the **acute toxicity study**, a 2x4 design will be used, consisting of 2 levels of stress (stressed and unstressed) and 4 levels of DU (control, low, mid and high). For stressed animals there will be daily routine stress with superimposed novel stress just prior to dosing (Hancock *et al.*). At several times after DU exposure (e.g., 1, 3, 7 and 30 days), brain samples will be analyzed for neurotransmitter levels, receptor numbers, evidence of oxidative stress and regional uranium concentrations. At the later time periods (e.g. 3, 7 and 30 days), samples will also be taken for neuropathological study. Neurobehavioral testing will be performed before dosing and weekly thereafter. The acute toxicity study will be performed in a number of experimental blocks. For the **long-term toxicity study**, a 2x4 design will be used with 2 levels of stress (stressed and unstressed) and 4 levels of implanted DU (tantalum negative control, low, medium, and high concentrations of DU pellets). Stress will be induced by frequently (such as 5 days/week) applying a routine stressor with superimposed periodic novel stress throughout the study (using parameters confirmed by a prior **pilot stress study**). Animals will be exposed to DU for 6 months. Behavioral testing will be performed before dosing and every 3 weeks thereafter. After 6 months, samples will be taken for neurochemical, neuropathological and brain uranium concentration analyses, as noted above. All determinations will be made on 3-5 separate samples. The study will be performed in experimental blocks.

The entire project will be conducted at two institutions, Virginia Tech (B. Jortner, principal investigator and neuropathologist and M. Ehrich, neurotoxicologist) and the University of Florida (D. Barber, heavy metal toxicologist), using the following temporal plan.

Months 1-4: Preparations for study (both institutions) and preliminary studies to identify appropriate doses (University of Florida). Consultation on doses (Virginia Tech).

Months 5-12 (kinetic study): Kinetics of DU (as uranyl acetate) in the brain and interaction of stress and DU are studied using 4 treatments (low DU, high DU, low DU + stress, and high DU + stress) at 4 times (e.g. 8 hours, 1 day, 7 days, and 30 days) after a single injection of DU (University of Florida). Data analysis (both institutions).

Months 13-26 (pilot stress study and acute toxicity study): A pilot study of long-term routine stress with periodic superimposed novel stress will be done, using plasma corticosterone levels as a measure. An acute study will be undertaken, assessing toxicity and uranium kinetics of a single intramuscular injection of DU (as uranyl acetate). This will employ 8 treatments (vehicle control, low DU, mid DU, high DU, control + stress, low DU + stress, mid DU + stress and high DU + stress) at 4 times (e.g. 1, 3, 7, and 30 days after injection). Procedures include: in-life study, neurobehavioral assessment, neuropathology (Virginia Tech); tissue uranium assays (University of Florida); neurochemistry, data analysis (both institutions).

Months 27-38 (acute toxicity study, long-term toxicity study): Analysis of samples collected from the acute study continues. In-life portion of long-term (6 month exposure) study is conducted, with assessment of toxicity and uranium kinetics following intramuscular injection of DU pellets. Eight treatments are used, tantalum control, low DU, medium DU, high DU, tantalum + stress, low DU + stress, medium DU + stress, and high DU + stress. Procedures include: in-life study, neurobehavioral assessment, neuropathology (Virginia Tech), uranium assays (University of Florida), neurochemistry, data analysis (both institutions).

Months 39-48 (acute and log-term toxicity studies, sample and data analysis): There is completion of brain uranium tissue assays (University of Florida), in-life portion of chronic study, neuropathology (Virginia Tech), neurochemistry (both institutions) from chronic and acute studies. Assembly of data and preparation of final report is done (both institutions).

Bernard S. Jortner, VMD	David Richardson, Director
Professor of Pathology, Principal Investigator	Virginia Tech Office of Sponsored Programs

Methods

1. Stress procedures- Stress was induced by a combination of four consecutive days of restraint stress followed by swim stress on the fifth day. For restraint stress, each animal was placed in an individual Plexiglas® tube (6 cm diameter X 22 cm long) (Konarska, Stewart and McCarty, 1989) with adequate breathing holes for a 30-minute period and then returned to its home cage. For swim stress, each animal was placed in a 4-chambered tank of water at 23°C - 25°C and allowed to swim for a 30-minute period. When swimming was completed, the animal was towel-dried for 1-2 minutes, placed under a heat lamp for an additional 2-3 minutes and returned to its home cage. Animals in the control (no stress) groups were handled daily by removing the animal from its home cage, placing it in a box and immediately returning it to its home cage. For the chronic study, animals are stressed in a random pattern of 1 day of handling, 1 day of swim (20 min. @ 22-24 C) and 3 days of restraint (30 min in a Plexiglas tube) for 22 weeks following the pellet implant procedure. Animals in treatment groups 1-4 (no stress exposure) are handled as described above.

2. Uranium analysis- Uranium analysis was performed by inductively coupled plasma-mass spectrometry (ICP-MS) on samples that had undergone nitric acid/peroxide digestion. Samples were placed in 15ml sealed glass pressure tubes with 0.5 ml of concentrated metal-free nitric acid (Optima, Fisher Scientific). Samples were heated to 140°C for 2 hours in a silicone oil bath, then 0.5ml of 30% hydrogen peroxide (Ultrex II, J.T. Baker) was added and samples heated at 110°C for a further 60 minutes. Samples were cooled, quantitatively transferred to acid-washed 5ml volumetric flasks, and brought to volume with deionized water (ElgaStat Maxima). Uranium analysis was performed by inductively coupled plasma-mass spectrometry (ICP-MS) by the Analytical Section of the Hazard Identification Core in the Southwest Hazardous Waste Program. Analysis was conducted on an HP 7500a ICP-MS using iridium as an internal standard. Uranium concentration was determined from a standard curve of uranium based on the m/z 238 signal. Five repetitions were performed per sample and the average used to calculate uranium concentration. The limit of quantitation of this method was 0.002ppb. Recovery was determined from samples spiked with 0.1-10ppb uranium and determined to be 96-108%. The limit of quantitation of this method under normal conditions was 0.01 ppb. By using a lower range of standards, the limit of quantitation was improved to 0.002ppb to accurately determined uranium concentrations from samples with low levels of uranium. Recovery was determined from samples spiked with 0.1-10ppb uranium and determined to be 96-108%.

3. Corticosterone determination- Blood was collected from the orbital sinus (under isofluorane anesthesia) immediately following the stress session. Whole blood was collected in heparinized microcentrifuge tubes, centrifuged @12000 rpm at 4° C for 4 minutes. The plasma was removed and frozen at -70° C until analysis by a corticosterone ^{125}I-radioimmunoassay kit (ICN Biomedicals, Costa Mesa, CA).

4. Neurobehavioral Evaluations-
The Functional Observational Battery (FOB) provides a range of clinical/behavioral evaluations, which include measures of behavioral and CNS excitability (home cage posture, activity rearing, arousal, ease of handling and removal from cage, involuntary movements, stereotypy, bizarre behavior); autonomic effects (salivation, lacrimation, urination, defecation, piloerection); effects on muscle tone and equilibrium (gait, fore-and hindlimb grip strengths, foot splay, duration/agility on rotarod); effects on motor and sensory systems (response to approach, touch, sound and tail pinch); and effects on the general physiology (weight, body temperature, respiration) (Moser *et al.*).

The horizontal motor activity level of animals is measured using an automated cage rack photobeam activity system. Motor activity is measured by counting interruptions of photobeams in a cage at 2-minute intervals over a period of 30 minutes. This system provides measurements of 1) non-ambulatory or fine movements by counting repeated breaks of the same photobeam, 2) ambulatory or larger locomotor movements by counting breaks of adjacent photobeams and 3) total motor activity by adding counts of all photobeam breaks.

Lastly, the passive-avoidance test is one that provides measures of learning and memory. Briefly, this involves a single acquisition trial session (training) on Day 0, prior to corticosterone dosing. The training consists of a 30-second adaptation in a darkened chamber prior to the trial onset. The trial begins with the onset of a bright light in the chamber containing the rat. To avoid the light, the rat can cross over into a darkened chamber at which time the animal is given a slight footshock for 3 seconds. The measurement recorded (latency) for each animal is the length of time taken to cross over to the darkened chamber. The maximum trial length is 180 seconds. The training trial is followed by a retention trial, which mimics training except no footshock is given. Once again, the recorded measurement is the length of time taken to cross over to the darkened chamber. The retention trials are performed at various intervals throughout the study period. Due to the placement of the training and retention trials in the study schedule, the passive avoidance measurements are largely a test of memory.

5. Pathology- Following perfusion-fixation with 4% paraformaldehyde and 0,2% glutaraldehyde, cross-sections of medulla, and spinal cord (cervical, thoracic and lumbar), peripheral nerve (sciatic, tibial, sural, vagus), optic nerve, and longitudinal sections of dorsal root ganglion and associated spinal nerve roots were embedded in Polybed epoxy resin, sectioned at 1 μm thickness and stained with toluidine blue and safranin for light microscopic study. Cross-sections of the frontal, parietal, and occipital levels of the cerebral hemispheres, midbrain, cerebellum and pons, and kidney and longitudinal section of the ocular globe (rostro-caudal plane) were embedded in a mixture of paraffin, sectioned at 5 μm thickness and stained with hematoxylin and eosin, Fluoro-Jade (a fluorescent stain to detect degenerating neurons- Schmeud and Hopkins), and immunostained for glial fibrillary acidic acid.

6. Neurochemistry- Half of the brain tissue (cerebellum, cortex, hippocampus and caudate putamen) from each animal was homogenized in 0.1M perchloric acid, centrifuged and the supernatant aliquoted into three tubes for the following analysis endpoints:
   a. HPLC determination of catecholamines (dopamine, serotonin and norepinephrine) (methods of Jussofie *et al.*, 1993).
   b. Glutathione (oxidized and reduced) determination
   c. Glutamate and GABA determinations (methods Yasumata *et al.*, 1998).

   The remaining half of the tissue regions was frozen at -70 C for subsequent determination of receptor numbers for regional neurotransmitters (D_1 and D_2 dopamine, serotonin 1a, GABA and acetylcholine receptors), using saturation radioligand binding.

7. Statistical Analysis- Responses measured each day of the pilot stress study were separately subjected to analysis of variance with mean separation by Bonferroni-corrected multiple comparisons. All calculations were performed using the SAS System (version 8.02, SAS Institute Inc. Cary, NC 27513).

8. Pellet Implant Procedure –
   Tantalum and depleted uranium pellet cleaning (sterile solutions and supplies are used). Pellets are sonicated in 0.1% Triton X-100 in a capped test tube for 5 minutes, drained into a metal basket, rinsed with 70% ethanol, soaked in 50% nitric acid for 3 minutes, rinsed with sterile water and poured onto filter paper in a glass petri dish. Using tweezers, pellets are placed in sterile vials with a small amount of 70% ethanol until implantation into muscle.

   Pellet Implant Procedure. Animal is anesthetized with isofluorane, the rear legs are shaved and prepared for surgery by washing with betadine, 70% ethanol and a final betadine scrub. The pellets are implanted using sterile procedure. A small (<0.5 inch) incision is made in the leg to reveal the gastrocnemius muscle. The skin is bluntly separated from the muscle using scissors. The pellets are removed from the vial, placed on filter paper in a petri dish and rinsed with sterile saline. A wire plunger is placed in the top of a 16-gauge needle and the pellets are loaded into the tip of the needle with tweezers. The tip of the needle is placed into the muscle (so that needle opening is not visible) and the plunger is depressed to insert the pellet. The pellets are aligned in the muscle in two rows of five. After all pellets are inserted, the incision is sutured. Each animals is monitored until it is able to walk about the cage.

DETAILED SUMMARY OF RAW MEANS AND P-VALUES FOR SELECTED RESPONSE VARIABLES FROM THE ACUTE DEPLETED URANIUM STUDY

FUNCTIONAL OBSERVATIONAL BATTERY

Table A1. P-values from analysis of FOB categorical response variables.
(note: No analysis is presented for response variables that did not change throughout the study; these include in cage posture, clonic and tonic movements, vocalizations, respirations, activity level; handling cage removal ease, lacrimation, salivation, piloerection, palpebral closure and reflex, oculocardiac response, pupil size and light response, visual placing, open field clonic and tonic movements, fecal, gait, gait score, mobility score, stereotypy, righting and tail-limb reflexes)

		Days after administration of DU			
Response	**Effect**	**6**	**13**	**20**	**27**
Handling: reactivity	Stress	0.7739	0.6392	0.9688	0.6546
	DU	0.4793	0.8964	0.4393	0.6612
	Stress*DU	0.9468	0.1868	0.6010	0.5198
Open field: arousal	Stress	0.0662	0.6996	0.9321	0.6423
	DU	0.3152	0.4776	0.1643	0.5771
	Stress*DU	0.4552	0.1676	**0.0423**	0.2750
Open field: rearing	Stress	0.1185	0.1907	0.1822	0.7164
	DU	0.4308	0.3088	0.6527	0.1744
	Stress*DU	0.6297	0.9005	0.3720	0.4365
Open field: urine pools	Stress	0.0922	0.6359	-	-
	DU	0.9811	0.5724	-	-
	Stress*DU	0.8098	0.6580	-	-
Open field: bizarre behavior	Stress	-	-	0.3129	0.9440
	DU	-	-	0.9641	0.1425
	Stress*DU	-	-	0.8283	0.6114
Approach response	Stress	0.3319	0.3502	0.9982	0.8648
	DU	0.6354	0.1898	0.1131	0.9570
	Stress*DU	0.6969	0.8191	0.1279	0.9243
Touch response	Stress	0.5493	0.9565	0.6931	0.7858
	DU	0.4197	0.1376	0.8932	0.4391
	Stress*DU	0.2520	0.2496	0.8676	0.6236
Click response	Stress	0.9543	0.0140	0.8755	0.7472
	DU	0.4077	0.4326	0.9762	**0.0452**
	Stress*DU	0.5749	0.0924	0.3478	0.2265
Pinch response	Stress	0.2821	0.8653	0.6783	0.6332
	DU	0.2959	0.7342	0.1189	0.6996
	Stress*DU	0.2226	**0.0148**	0.2292	**0.0310**
Rotarod agility score	Stress	0.3162	0.4691	0.8799	0.5638
	DU	0.6897	0.6687	0.6703	0.4080
	Stress*DU	0.9880	0.8796	0.4716	0.1655

Table A2. P-values from analysis of FOB continuous response variables.

Effect	Response variable					
	Splay	Hindgrip	Foregrip	Rota_Sec	Temperature	Weight
Stress	0.7276	0.1974	0.1159	0.6954	0.7773	0.5562
DU	0.7494	0.1232	**0.0021**	0.4621	0.2131	**<0.0001**
Stress*DU	0.7536	0.6653	0.2249	0.1805	0.9013	0.4732
Day	**<0.0001**	**<0.0001**	**<0.0001**	0.1831	**<0.0001**	**<0.0001**
Stress x Day	0.4944	0.9103	0.8232	0.8575	0.4622	0.5323
DU x Day	0.7895	0.8534	0.3014	0.9676	**0.00016**	**<0.0001**
Stress x DU x Day	0.7989	0.3667	0.6451	0.6121	0.4597	0.4700

MOTOR ACTIVITY

Table A3. P-values from repeated measure analysis of variance of motor activity responses.

Effect	Ambulations	Non-ambulatory	Total
Stress	0.8205	0.3221	0.5889
Dose	**0.0078**	0.1613	0.0556
Stress x Dose	0.7126	0.1959	0.4197
DAY	0.0799	0.2935	0.1487
Stress x Day	0.9608	0.7854	0.8405
Dose x Day	0.5146	0.3112	0.2916
Stress x Dose x Day	0.8126	0.6864	0.9426

Table A4. Motor activity responses. Values are expressed as beam breaks. Each value is the mean ± SEM (based on the pooled MSE).

Days after administration	Stress	DU Dose	Ambulatory	Non-ambulatory	Total
6	Non-stressed	0	131.2 ± 8.56	244.2 ± 15.9	374.7 ± 22.65
		0.1	122.3 ± 10.46	265.6 ± 19.49	354.8 ± 23.61
		0.3	99.8 ± 8.59	238.5 ± 15.9	351.0 ± 22.65
		1	88.5 ± 8.54	189.5 ± 15.9	324.5 ± 22.65
	Stressed	0	128.4 ± 8.55	279.6 ± 15.96	388.9 ± 27.74
		0.1	109.7 ± 10.51	227.3 ± 19.65	362.3 ± 27.74
		0.3	106.1 ± 8.62	244.0 ± 15.94	349.5 ± 27.74
		1	98.1 ± 8.56	225.6 ± 15.96	343.5 ± 27.74
13	Non-stressed	0	121.8 ± 8.89	233.4 ± 16.63	339.8 ± 22.65
		0.1	113.8 ± 10.46	247.5 ± 19.49	343.2 ± 22.65
		0.3	102.1 ± 8.59	239.5 ± 15.9	349.0 ± 22.65
		1	97.6 ± 9.07	213.5 ± 17.07	330.2 ± 22.65
	Stressed	0	117.2 ± 8.7	247.0 ± 16.3	277.5 ± 22.64
		0.1	106.5 ± 10.51	228.3 ± 19.65	310.3 ± 24.18
		0.3	107.8 ± 8.77	267.5 ± 16.27	334.1 ± 22.64
		1	98.5 ± 8.71	242.9 ± 16.3	297.3 ± 22.64
20	Non-stressed	0	124.6 ± 8.56	227.0 ± 15.9	408.8 ± 22.73
		0.1	102.5 ± 10.46	246.0 ± 19.49	364.9 ± 23.18
		0.3	103.9 ± 8.59	243.6 ± 15.9	365.7 ± 22.73
		1	105.6 ± 8.54	229.0 ± 15.9	359.9 ± 22.73
	Stressed	0	108.6 ± 8.55	256.3 ± 15.96	335.9 ± 28.03
		0.1	107.0 ± 10.51	211.2 ± 19.65	333.7 ± 28.03
		0.3	108.7 ± 8.62	254.0 ± 15.94	317.1 ± 28.03
		1	100.7 ± 8.56	231.1 ± 15.96	325.4 ± 28.03
27	Non-stressed	0	109.6 ± 8.56	215.6 ± 15.9	349.6 ± 22.78
		0.1	108.5 ± 10.46	234.0 ± 19.49	375.0 ± 23.22
		0.3	93.9 ± 8.59	234.7 ± 15.9	362.2 ± 22.78
		1	93.0 ± 8.54	204.9 ± 15.9	328.4 ± 22.78
	Stressed	0	109.7 ± 8.55	249.4 ± 15.96	323.1 ± 22.75
		0.1	97.3 ± 10.51	229.1 ± 19.65	341.0 ± 23.19
		0.3	106.6 ± 8.62	222.3 ± 15.94	331.2 ± 22.75
		1	90.5 ± 8.56	228.0 ± 15.96	317.9 ± 22.75

CATECHOLAMINES

Serotonin

Table A5. P-values from analysis of variance performed on measurements of serotonin content in various brain regions.

Days after administration	Effect	Brain Region			
		Cerebral Cortex	Cerebellum	Caudate putamen	Hippocampus
1	DU Dose	0.7039	0.6731	0.7172	0.0833
	Stress	0.3417	0.5557	0.5303	0.7399
	DU Dose x Stress	0.8767	0.5101	0.0556	0.5313
3	DU Dose	**0.0435**	0.6358	0.4269	0.4177
	Stress	0.3998	0.1498	0.7940	0.6826
	DU Dose x Stress	0.1850	0.6899	0.6475	0.8072
7	DU Dose	0.2216	0.1150	**0.0016**	**0.0378**
	Stress	0.7774	0.4762	**0.0101**	0.7506
	DU Dose x Stress	0.9414	0.8802	0.4101	0.7435
30	DU Dose	0.4143	0.8848	0.9638	0.1700
	Stress	0.4157	0.7028	0.8282	0.6820
	DU Dose x Stress	0.3577	0.6443	0.3169	0.5094

Table A6. Mean serotonin content for the cerebral cortex by stress treatment, DU dose and days after administration of depleted uranium[z].

Stress	DU dose	Days after administration			
		1	3	7	30
Non-stressed	0	1.029 ± 0.098	0.942 ± 0.125	1.229 ± 0.089	1.477 ± 0.820
	0.1	1.025 ± 0.090	1.402 ± 0.115	1.133 ± 0.089	1.726 ± 0.820
	0.3	1.042 ± 0.098	1.046 ± 0.125	1.020 ± 0.100	1.608 ± 0.917
	1	1.065 ± 0.098	0.977 ± 0.125	1.147 ± 0.089	1.330 ± 0.820
Stressed	0	0.929 ± 0.098	0.986 ± 0.125	1.232 ± 0.089	3.799 ± 0.820
	0.1	0.897 ± 0.098	1.190 ± 0.125	1.063 ± 0.089	1.455 ± 0.820
	0.3	1.051 ± 0.090	1.353 ± 0.115	1.055 ± 0.089	1.378 ± 0.820
	1	1.029 ± 0.098	1.127 ± 0.125	1.107 ± 0.089	1.456 ± 0.820

[z] Activity is expressed as ng/mg tissue. Each value is the mean of 15-22 observations ± the standard error based on the pooled mean square error.

Table A7. Mean serotonin content for the cerebellum by stress treatment, DU dose and days after administration of depleted uranium[z].

		Days after administration			
Stress	**DU dose**	**1**	**3**	**7**	**30**
Non-stressed	0	0.588 ± 0.172	0.189 ± 0.022	0.117 ± 0.032	0.170 ± 0.037
	0.1	0.281 ± 0.173	0.145 ± 0.02	0.109 ± 0.037	0.166 ± 0.037
	0.3	0.069 ± 0.385	0.184 ± 0.02	0.171 ± 0.029	0.157 ± 0.039
	1	0.138 ± 0.223	0.161 ± 0.022	0.154 ± 0.027	0.166 ± 0.035
Stressed	0	0.157 ± 0.193	0.147 ± 0.02	0.147 ± 0.027	0.148 ± 0.037
	0.1	0.090 ± 0.273	0.150 ± 0.02	0.124 ± 0.029	0.140 ± 0.037
	0.3	0.212 ± 0.173	0.153 ± 0.02	0.177 ± 0.029	0.183 ± 0.035
	1	0.225 ± 0.172	0.142 ± 0.02	0.150 ± 0.027	0.164 ± 0.035

[z] Activity is expressed as ng/mg tissue. Each value is the mean of 15-22 observations ± the standard error based on the pooled mean square error.

Table A8. Mean serotonin content for the caudate putamen by stress treatment, DU dose and days after administration of depleted uranium[z].

		Days after administration			
Stress	**DU dose**	**1**	**3**	**7**	**30**
Non-stressed	0	1.356 ± 0.267	0.913 ± 0.303	0.548 ± 0.166	1.152 ± 0.418
	0.1	1.055 ± 0.259	1.048 ± 0.308	0.377 ± 0.156	1.861 ± 0.418
	0.3	1.341 ± 0.267	0.918 ± 0.325	0.700 ± 0.166	1.304 ± 0.418
	1	1.131 ± 0.267	0.999 ± 0.303	0.791 ± 0.156	1.278 ± 0.418
Stressed	0	0.934 ± 0.267	0.768 ± 0.303	0.802 ± 0.156	1.782 ± 0.418
	0.1	1.460 ± 0.267	0.994 ± 0.303	0.619 ± 0.166	1.147 ± 0.418
	0.3	1.268 ± 0.259	0.911 ± 0.308	0.702 ± 0.166	1.349 ± 0.418
	1	1.516 ± 0.267	1.366 ± 0.303	1.183 ± 0.156	1.541 ± 0.418

[z] Activity is expressed as ng/mg tissue. Each value is the mean of 15-22 observations ± the standard error based on the pooled mean square error.

Table A9. Mean serotonin content for the hippocampus by stress treatment, DU dose and days after administration of depleted uranium[z].

		Days after administration			
Stress	**DU dose**	**1**	**3**	**7**	**30**
Non-stressed	0	0.424 ± 0.163	0.424 ± 0.260	0.364 ± 0.156	0.495 ± 0.193
	0.1	0.707 ± 0.152	0.473 ± 0.179	0.258 ± 0.141	0.771 ± 0.176
	0.3	0.936 ± 0.147	0.581 ± 0.193	0.509 ± 0.156	0.740 ± 0.193
	1	0.629 ± 0.163	0.695 ± 0.176	0.631 ± 0.128	0.819 ± 0.176
Stressed	0	0.528 ± 0.163	0.476 ± 0.218	0.258 ± 0.156	0.445 ± 0.217
	0.1	0.787 ± 0.183	0.488 ± 0.176	0.377 ± 0.141	0.550 ± 0.192
	0.3	0.728 ± 0.166	0.795 ± 0.179	0.419 ± 0.141	0.969 ± 0.176
	1	0.783 ± 0.163	0.612 ± 0.176	0.604 ± 0.141	0.670 ± 0.176

[z] Activity is expressed as ng/mg tissue. Each value is the mean of 15-22 observations ± the standard error based on the pooled mean square error.

Norepinephrine

Table A10. P-values from analysis of variance performed on the measurements of norepinephrine content in various brain regions.

		Brain Region			
Days after administration	Effect	Cerebral Cortex	Cerebellum	Caudate putamen	Hippocampus
1	DU Dose	0.8047	0.4314	0.3226	0.4201
	Stress	0.4608	0.6970	0.4293	0.6750
	DU Dose x Stress	0.7757	0.5713	0.2458	0.4243
3	DU Dose	0.2939	0.5123	0.5439	0.2448
	Stress	0.2560	0.9000	0.9808	0.4124
	DU Dose x Stress	0.3297	0.7824	0.6257	0.5639
7	DU Dose	0.4637	0.7382	0.0990	0.1038
	Stress	0.7938	0.5065	0.2539	0.2331
	DU Dose x Stress	0.7599	0.6388	0.3194	0.3874
30	DU Dose	0.6874	0.6955	0.8803	0.1742
	Stress	0.7300	0.2410	0.1212	0.5497
	DU Dose x Stress	0.4906	0.3938	0.5430	0.3728

Table A11. Mean norepinephrine content for the cerebral cortex by stress treatment, DU dose and days after administration of depleted uranium[z].

		Days after administration			
Stress	DU dose	1	3	7	30
Non-stressed	0	1.993 ± 0.193	2.029 ± 0.318	2.200 ± 0.193	2.806 ± 0.535
	0.1	2.129 ± 0.183	2.723 ± 0.295	1.877 ± 0.193	3.190 ± 0.535
	0.3	1.975 ± 0.193	2.026 ± 0.318	2.211 ± 0.193	2.917 ± 0.535
	1	2.133 ± 0.193	1.852 ± 0.318	2.046 ± 0.193	2.424 ± 0.535
Stressed	0	2.265 ± 0.193	2.278 ± 0.318	2.147 ± 0.193	2.888 ± 0.535
	0.1	2.111 ± 0.193	2.373 ± 0.318	1.983 ± 0.193	2.633 ± 0.535
	0.3	2.038 ± 0.183	2.743 ± 0.295	2.001 ± 0.193	2.782 ± 0.535
	1	2.137 ± 0.193	2.192 ± 0.318	2.088 ± 0.193	2.747 ± 0.535

[z] Activity is expressed as ng/mg tissue. Each value is the mean of 15-22 observations ± the standard error based on the pooled mean square error.

Table A12. Mean norepinephrine content for the cerebellum by stress treatment, DU dose and days after administration of depleted uranium[z]..

Stress	DU dose	Days after administration 1	3	7	30
Non-stressed	0	1.437 ± 0.210	1.432 ± 0.189	1.227 ± 0.143	0.931 ± 0.151
	0.1	1.350 ± 0.203	1.415 ± 0.191	1.113 ± 0.143	0.888 ± 0.151
	0.3	1.127 ± 0.210	1.429 ± 0.189	1.201 ± 0.143	0.869 ± 0.151
	1	1.105 ± 0.210	1.272 ± 0.189	1.272 ± 0.143	0.984 ± 0.151
Stressed	0	1.379 ± 0.210	1.551 ± 0.189	1.142 ± 0.143	0.905 ± 0.151
	0.1	1.213 ± 0.210	1.360 ± 0.189	1.117 ± 0.143	0.948 ± 0.151
	0.3	1.306 ± 0.203	1.334 ± 0.191	1.271 ± 0.143	1.092 ± 0.151
	1	1.277 ± 0.210	1.346 ± 0.189	1.076 ± 0.143	0.989 ± 0.151

[z] Activity is expressed as ng/mg tissue. Each value is the mean of 15-22 observations ± the standard error based on the pooled mean square error.

Table A13. Mean norepinephrine content for the caudate putamen by stress treatment, DU dose and days after administration of depleted uranium[z]..

Stress	DU dose	Days after administration 1	3	7	30
Non-stressed	0	1.394 ± 0.464	1.313 ± 0.399	0.886 ± 0.332	0.903 ± 0.524
	0.1	1.595 ± 0.453	1.569 ± 0.405	1.022 ± 0.332	0.930 ± 0.524
	0.3	2.035 ± 0.464	1.411 ± 0.399	1.243 ± 0.332	1.201 ± 0.572
	1	1.545 ± 0.464	2.119 ± 0.399	1.514 ± 0.332	1.143 ± 0.524
Stressed	0	1.572 ± 0.464	1.837 ± 0.399	1.462 ± 0.332	2.098 ± 0.572
	0.1	1.640 ± 0.464	1.507 ± 0.399	1.211 ± 0.332	1.643 ± 0.524
	0.3	1.689 ± 0.453	1.352 ± 0.405	1.022 ± 0.332	1.357 ± 0.524
	1	2.234 ± 0.464	1.690 ± 0.399	1.656 ± 0.332	1.122 ± 0.524

[z] Activity is expressed as ng/mg tissue. Each value is the mean of 15-22 observations ± the standard error based on the pooled mean square error.

Table A14. Mean norepinephrine content for the hippocampus by stress treatment, DU dose and days after administration of depleted uranium[z]..

Stress	DU dose	Days after administration 1	3	7	30
Non-stressed	0	2.623 ± 0.378	3.337 ± 0.404	2.939 ± 0.452	3.071 ± 0.678
	0.1	3.017 ± 0.366	3.397 ± 0.410	3.394 ± 0.452	2.801 ± 0.678
	0.3	3.202 ± 0.378	3.254 ± 0.404	3.072 ± 0.452	3.258 ± 0.678
	1	3.105 ± 0.378	2.568 ± 0.404	4.324 ± 0.452	3.522 ± 0.678
Stressed	0	2.933 ± 0.378	3.161 ± 0.404	3.321 ± 0.452	2.590 ± 0.678
	0.1	2.714 ± 0.378	3.308 ± 0.404	2.786 ± 0.452	2.843 ± 0.678
	0.3	2.838 ± 0.366	3.689 ± 0.410	2.875 ± 0.452	4.895 ± 0.678
	1	3.185 ± 0.378	3.133 ± 0.404	3.419 ± 0.452	3.414 ± 0.678

[z] Activity is expressed as ng/mg tissue. Each value is the mean of 15-22 observations ± the standard error based on the pooled mean square error.

Dopamine

Table A15. P-values from analysis of variance performed on measurements of dopamine content in various brain regions.

Days after administration	Effect	Brain Region: Cerebral cortex	Cerebellum	Caudate putamen	Hippocampus
1	DU Dose	0.4948	0.2963	0.3463	0.9382
	Stress	0.4559	0.4105	0.8444	0.8102
	DU Dose x Stress	0.8530	0.3208	0.9626	0.5533
3	DU Dose	0.4013	0.0647	**0.0396**	0.7112
	Stress	0.3478	0.8394	0.1881	0.1925
	DU Dose x Stress	0.3871	0.4135	**0.0511**	0.6643
7	DU Dose	0.2770	0.2458	0.9544	0.1819
	Stress	0.6826	0.4095	0.3134	0.2858
	DU Dose x Stress	0.9641	0.4483	0.4943	0.6133
30	DU Dose	0.4498	0.0825	**0.0107**	0.0990
	Stress	0.4676	0.8382	0.6899	0.3614
	DU Dose x Stress	0.5248	0.5570	0.1094	0.4150

Table A16. Mean dopamine content for the cerebral cortex by stress treatment, DU dose and days after administration of depleted uranium.[z]

Stress	DU dose	Days after administration: 1	3	7	30
Non-stressed	0	0.984 ± 0.466	0.397 ± 2.817	0.989 ± 0.481	0.890 ± 0.743
	0.1	0.646 ± 0.451	0.614 ± 2.573	0.592 ± 0.481	1.082 ± 0.743
	0.3	0.853 ± 0.466	0.644 ± 2.817	0.423 ± 0.481	2.649 ± 0.743
	1	1.313 ± 0.466	8.549 ± 2.817	1.304 ± 0.481	1.590 ± 0.743
Stressed	0	0.899 ± 0.466	0.382 ± 2.817	0.594 ± 0.481	1.009 ± 0.743
	0.1	0.728 ± 0.466	0.792 ± 2.817	0.443 ± 0.481	1.176 ± 0.743
	0.3	0.653 ± 0.451	0.957 ± 2.573	0.487 ± 0.481	1.143 ± 0.743
	1	0.890 ± 0.466	0.642 ± 2.817	1.252 ± 0.481	1.557 ± 0.743

[z] Activity is expressed as ng/mg tissue. Each value is the mean of 15-22 observations ± the standard error based on the pooled mean square error.

Table A17. Mean dopamine content for the cerebellum by stress treatment, DU dose and days after administration of depleted uranium.[z].

Stress	DU dose	Days after administration			
		1	3	7	30
Non-stressed	0	0.221 ± 0.064	0.108 ± 0.020	0.109 ± 0.025	0.065 ± 0.022
	0.1	0.196 ± 0.064	0.087 ± 0.020	0.108 ± 0.020	0.098 ± 0.022
	0.3	0.066 ± 0.064	0.069 ± 0.020	0.094 ± 0.020	0.100 ± 0.022
	1	0.034 ± 0.064	0.077 ± 0.020	0.078 ± 0.020	0.092 ± 0.022
Stressed	0	0.108 ± 0.045	0.090 ± 0.020	0.105 ± 0.020	0.071 ± 0.022
	0.1	0.072 ± 0.064	0.111 ± 0.022	0.102 ± 0.022	0.080 ± 0.022
	0.3	0.135 ± 0.045	0.068 ± 0.022	0.140 ± 0.022	0.091 ± 0.021
	1	0.055 ± 0.064	0.081 ± 0.020	0.085 ± 0.022	0.106 ± 0.022

[z] Activity is expressed as ng/mg tissue. Each value is the mean of 15-22 observations ± the standard error based on the pooled mean square error.

Table A18. Mean dopamine content for the caudate putamen by stress treatment, DU dose and days after administration of depleted uranium.[z]

Stress	DU dose	Days after administration			
		1	3	7	30
Non-stressed	0	53.7 ± 5.7	54.5 ± 5.8	68.8 ± 8.3	73.2 ± 9.7
	0.1	54.1 ± 5.4	61.8 ± 5.8	60.5 ± 8.3	95.7 ± 9.7
	0.3	48.3 ± 5.7	64.0 ± 5.8	59.6 ± 8.3	67.0 ± 9.7
	1	48.5 ± 5.7	**32.8 ± 5.8**	66.6 ± 8.3	68.9 ± 9.7
Stressed	0	57.0 ± 5.7	56.8 ± 5.8	57.1 ± 8.3	93.6 ± 9.7
	0.1	52.8 ± 5.7	60.1 ± 5.8	60.3 ± 8.3	78.1 ± 9.7
	0.3	49.2 ± 5.4	59.4 ± 5.8	63.2 ± 8.3	51.8 ± 9.7
	1	48.2 ± 5.7	**58.9 ± 5.8**	59.2 ± 8.3	71.6 ± 9.7

[z] Activity is expressed as ng/mg tissue. Each value is the mean of 15-22 observations ± the standard error based on the pooled mean square error.

Table A19. Mean dopamine content for the hippocampus by stress treatment, DU dose and days after administration of depleted uranium.[z]

Stress	DU dose	Days after administration			
		1	3	7	30
Non-stressed	0	0.204 ± 0.713	0.259 ± 0.093	0.292 ± 0.089	0.050 ± 0.110
	0.1	0.779 ± 0.319	0.333 ± 0.093	0.277 ± 0.077	0.296 ± 0.057
	0.3	0.194 ± 0.504	0.246 ± 0.080	0.236 ± 0.077	0.291 ± 0.057
	1	0.219 ± 0.411	0.255 ± 0.080	0.204 ± 0.069	0.260 ± 0.057
Stressed	0	0.205 ± 0.411	0.144 ± 0.093	0.322 ± 0.089	0.215 ± 0.057
	0.1	0.221 ± 0.411	0.219 ± 0.073	0.424 ± 0.077	0.233 ± 0.065
	0.3	0.583 ± 0.411	0.282 ± 0.083	0.344 ± 0.069	0.363 ± 0.057
	1	0.707 ± 0.411	0.195 ± 0.067	0.163 ± 0.077	0.256 ± 0.065

[z] Activity is expressed as ng/mg tissue. Each value is the mean of 15-22 observations ± the standard error based on the pooled mean square error.

GAMMA AMINOBUTYRIC ACID (GABA)

Table A20. GABA concentrations (nmol GABA/g tissue) in rat brain regions following treatment with uranium and/or stress. Values are presented as the mean of 5 observations ± SD.

Cortex

		Days after administration			
Stress	**DU dose**	**1**	**3**	**7**	**30**
Non-stressed	0	1212 ± 148	1286 ± 341	1544 ± 407	1331 ± 159
	0.1	1243 ± 149	1324 ± 405	1348 ± 276	1486 ± 246
	0.3	1295 ± 391	1319 ± 288	1496 ± 171	1366 ± 374
	1	1085 ± 210	1441 ± 122	1422 ± 251	1479 ± 118
Stressed	0	1360 ± 187	1375 ± 252	1315 ± 196	1541 ± 164
	0.1	1244 ± 285	1312 ± 178	1375 ± 197	1370 ± 32
	0.3	1082 ± 292	1493 ± 284	1435 ± 114	1455 ± 92
	1	1171 ± 252	1102 ± 431	1310 ± 285	1509 ± 83

Cerebellum

		Days after administration			
Stress	**DU dose**	**1**	**3**	**7**	**30**
Non-stressed	0	1089 ± 379	1493 ± 419	1499 ± 154	1496 ± 159
	0.1	1159 ± 332	1569 ± 242	1382 ± 235	1537 ± 109
	0.3	1167 ± 284	1385 ± 364	1517 ± 134	2004 ± 898
	1	1247 ± 277	1339 ± 274	1393 ± 189	1689 ± 110
Stressed	0	1373 ± 278	1517 ± 301	1528 ± 239	1690 ± 185
	0.1	1297 ± 251	1946 ± 1276	1542 ± 170	1647 ± 244
	0.3	1370 ± 347	1549 ± 100	1576 ± 173	1717 ± 116
	1	1427 ± 206	1476 ± 415	1525 ± 189	1580 ± 212

Hippocampus

Stress	DU dose	Days after administration 1			3			7			30		
Non-stressed	0	2128	±	414	2217	±	688	2224	±	319	2622	±	272
	0.1	1862	±	641	2287	±	519	1935	±	423	2348	±	569
	0.3	2106	±	261	2179	±	607	2105	±	350	2460	±	304
	1	1690	±	444	2140	±	560	2895	±	477	2249	±	261
Stressed	0	1673	±	539	2255	±	719	2120	±	749	2386	±	525
	0.1	2309	±	444	2527	±	842	1944	±	249	2704	±	162
	0.3	1863	±	196	2186	±	210	2236	±	328	2545	±	782
	1	2162	±	424	2347	±	962	2242	±	536	2365	±	507

Caudate Putamen

Stress	DU dose	Days after administration 1			3			7			30		
Non-stressed	0	1594	±	616	1655	±	400	1584	±	399	1736	±	378
	0.1	1709	±	157	2105	±	316	1845	±	407	2019	±	492
	0.3	1761	±	368	2208	±	456	1831	±	432	2157	±	166
	1	1956	±	665	1941	±	963	1908	±	306	1859	±	390
Stressed	0	1929	±	740	1791	±	521	1764	±	293	2033	±	378
	0.1	1541	±	252	1961	±	497	1927	±	487	2002	±	527
	0.3	1761	±	152	2281	±	248	1779	±	234	1797	±	563
	1	1830	±	263	2142	±	675	1993	±	94	1924	±	99

REDUCED GLUTATHIONE

Table A21. Reduced glutathione (μmol GSH/g tissue) in rat brain regions following treatment with uranium and/or stress. Values are presented as the mean of 3 observations.

Cortex

Stress	DU dose	Days after administration 1	3	7	30
Non-stressed	0	0.92	1.08	1.02	0.76
	0.1	1.02	1.38	1.10	0.89
	0.3	0.83	1.15	0.98	0.78
	1	0.61	1.40	0.86	0.95
Stressed	0	0.95	1.34	1.23	0.88
	0.1	0.62	1.31	1.06	0.79
	0.3	0.94	1.07	1.15	0.89
	1	0.49	1.33	0.97	0.85

Cerebellum

Stress	DU dose	Days after administration 1	3	7	30
Non-stressed	0	0.86	1.77	0.93	1.09
	0.1	0.82	1.65	1.05	1.17
	0.3	0.48	1.58	0.86	1.01
	1	1.04	1.29	0.94	1.09
Stressed	0	0.77	1.69	1.08	1.05
	0.1	0.91	1.71	1.11	1.09
	0.3	0.86	1.54	1.27	1.08
	1	0.79	1.47	1.07	1.12

Hippocampus

Stress	DU dose	Days after administration 1	3	7	30
Non-stressed	0	1.02	1.76	1.27	1.12
	0.1	1.04	1.59	1.68	1.12
	0.3	0.93	1.34	1.54	1.24
	1	1.24	1.42	1.24	1.42
Stressed	0	0.93	1.81	1.42	1.42
	0.1	0.91	1.84	1.41	1.11
	0.3	1.21	1.63	1.26	1.26
	1	1.19	1.41	1.10	1.12

Caudate Putamen

Stress	DU dose	Days after administration 1	3	7	30
Non-stressed	0	1.13	1.27	1.01	1.43
	0.1	1.09	1.23	1.02	1.36
	0.3	1.16	1.07	0.92	1.28
	1	0.92	1.09	1.12	1.40
Stressed	0	0.93	1.55	1.14	1.38
	0.1	1.17	1.33	1.18	1.25
	0.3	1.18	1.45	1.02	1.33
	1	1.10	1.24	0.99	1.28

CLINICAL CHEMISTRY

Table A22.

Serum Urea Nitrogen, mg/dl

Day 1			Day 3			Day 7			Day 30		
Group	Mean	SD	Group	Mean	SD	Group	Mean	SD	Group	Mean	SD
1	22.000	3.317	1	22.600	1.520	1	23.200	3.421	1	25.800	3.564
2	22.200	0.837	2	35.667*	6.154	2	25.600	4.720	2	26.200	5.020
3	24.400	3.435	3	46.000*	3.536	3	55.200	36.930	3	23.200	1.095
4	24.200	4.868	4	56.000*	6.042	4	104.800*	29.120	4	27.000	2.550
5	22.000	2.582	5	23.800	1.095	5	23.400	1.820	5	22.600	1.140
6	23.250	2.062	6	36.800*	4.025	6	27.200	1.640	6	23.600	1.342
7	23.667	3.445	7	50.167*	4.956	7	62.200	21.940	7	26.600	3.647
8	25.000	1.414	8	61.000*	11.554	8	110.000*	44.470	8	26.800	2.387

*different from control (Group 1), $p < 0.05$, three way ANOVA

Group 1- negative control, 2- 0.1 mg/kg DU, 3- 0.3 mg/kg DU, 4- 1.0 mg/kg DU, 5- stress, 6- stress + 0.1 mg/kg DU, 7- stress + 0.3 mg/kg DU. 8- stress + 1.0 mg/kg DU

Serum Creatinine, mg/dl

Day 1			Day 3			Day 7			Day 30		
Group	Mean	SD	Group	Mean	SD	Group	Mean	SD	Group	Mean	SD
1	0.38000	0.04472	1	0.4000	0.0000	1	0.3800	0.0447	1	0.3800	0.04472
2	0.40000	0.00000	2	0.6500	0.0837	2	0.4200	0.0447	2	0.4200	0.04472
3	0.44000	0.05477	3	1.0000*	0.2345	3	0.9800	0.7981	3	0.4000	0.00000
4	0.46000*	0.05477	4	1.6600*	0.2881	4	2.2200*	0.5310	4	0.4000	0.00000
5	0.37500	0.05000	5	0.4200	0.0447	5	0.3800	0.0447	5	0.4000	0.00000
6	0.40000	0.00000	6	0.6600	0.0894	6	0.4600	0.0548	6	0.4000	0.00000
7	0.40000	0.00000	7	1.0833*	0.1329	7	1.1000	0.3742	7	0.4200	0.04472
8	0.46000*	0.05477	8	1.7800*	0.1304	8	2.3600*	1.0644	8	0.4600*	0.05477

*different from control (Group 1), $p < 0.05$, three way ANOVA

Group 1- negative control, 2- 0.1 mg/kg DU, 3- 0.3 mg/kg DU, 4- 1.0 mg/kg DU, 5- stress, 6- stress + 0.1 mg/kg DU, 7- stress + 0.3 mg/kg DU. 8- stress + 1.0 mg/kg DU

ABSTRACTS

Abstract of paper presented at the 2003 Meeting of Society for Neuroscience

STRESS AND NEUROTOXICANT EXPOSURE. COMPARISON OF THE EFFECTS OF SEVERAL STRESS MODELS ON THE ACUTE NEUROTOXICITY OF THE ORGANOPHOSPHATE CHLORPYRIFOS. S.K. Hancock, M.F. Ehrich, J. Hinckley, T. Pung, K.L. Farris, B.S. Jortner*. *Laboratory for Neurotoxicity Studies, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech, Blacksburg, VA, USA*

Considerable interest exists regarding the possible role of stress in modifying neurotoxicity. We have evaluated several stress models in Sprague-Dawley rats, using serial measures such as body weight, selected neurobehavioral tests and plasma corticosterone concentration (samples were collected within 10 minutes after the stress episode). We then assessed the effect of these stressors on the ability of a neurotoxic organophosphate (the insecticide chlorpyrifos) to inhibit brain acetylcholinesterase. The stress models were (all administered 5 days/week for 6 weeks, n=8/group): Group 1- handling (control); Group 2- restraint for 30 minutes; Group 3- 4 days restraint for 30 minutes, followed by 1 day swimming for 30 minutes; Group 4- swimming for 30 minutes. On day 39 the rats were bled for plasma true acetylcholinesterase (AChE) determination, and then 1/2 the rats in each group (n=4) were administered 60 mg/kg chlorpyrifos subcutaneously. The rats were sacrificed on day 43, and the brains collected for AChE determination. The major stress related findings during the 6-week exposure period included elevated plasma corticosterone. in Groups 3 (restraint/swim) and 4 compared to the controls and Group 2 (restraint) ($p< 0.05$). This was noted from day 4 onward. These levels of corticosterone did not differ significantly between groups 3 and 4. All stress groups had increase in fine motor movements on days 38 and 42. Stress had no effect on plasma AChE levels determined on day 39. Chlorpyrifos administered on that day significantly ($p< 0.05$) inhibited the activity of brain AChE in the control and all 3 stress groups, with no significant differences among them. Thus, none of our stress states modified the intracerebral anticholinesterase effect of 60 mg/kg chlorpyrifos in rats. (Supported by DAMD17-01-1-0775, US Army Medical Research and Materiel Command)

Abstract of paper presented at the 2004 Bioscience Review.

The Effect of Stress on the Acute Neurotoxicity of the Organophosphate Chlorpyrifos

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The potential of stress to modify the anti-cholinesterase effects of nerve agents is a matter of interest to military and civilian defense planners, since it is a likely component of both combat operations and terrorist attacks. To help address this question, we employed several stress models and assessed their effects on the ability of a neurotoxic organophosphate (the insecticide chlorpyrifos) to inhibit brain acetylcholinesterase (AChE). Stress was developed in male Sprague-Dawley rats over a prolonged period, and was serially measured by body weight, selected neurobehavioral tests and plasma corticosterone concentrations (samples were collected 10 minutes after the stress episode). The stress models (all administered 5 days/week for 6 weeks) were: Group 1 – handling (control); Group 2 – restraint for 30 minutes; Group 3 – four days restraint for 30 minutes, followed by one day swimming for 30 minutes; Group 4 – swimming for 30 minutes (n=8/group). On day 32 (note- the first day of the study is day 0) the rats were bled for blood AChE determination, and on day 39 one-half of the rats in each group (n=4) were administered 60 mg/kg chlorpyrifos subcutaneously. The rats were sacrificed on day 43, and the brains collected for AChE determination. The major stress related findings during the 6-week exposure period included elevated plasma corticosterone in Group 3 (restraint/swim) and Group 4 compared to the controls and Group 2 (restraint) ($p<0.05$). This was noted from day 4 onward. These levels of corticosterone did not differ significantly between Group 3 and Group 4. By day 18, body weight was less in Group 4 than in the other groups. Stress had no effect on blood AChE levels determined on day 32. Chlorpyrifos administered on day 39 significantly ($p<0.05$) inhibited the activity of brain AChE in the control and all 3 stress groups, with no significant difference among them. Thus none of the stress states modified the intracerebral anticholinesterase effect of 60 mg/kg chlorpyrifos in rats. (This was supported by the US Army Medical Research and Materiel Command grants DAMD17-01-1-0775 and DAMD17-99-1-9489,)

Abstracts accepted for the 2005 Society of Toxicology annual meeting.

NEUROLOGICAL EFFECTS OF ACUTE URANIUM EXPOSURE

Barber, DS[1], Hancock, SK[2], McNally, AM[1], Hinckley, J[2], Binder, E[2], Ehrich, MF[2] and Jortner, BS[2].

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We have previously shown that a single intraperitoneal exposure to depleted uranium increased regional brain uranium content. In the current study, we examined the neurological effects of a single intramuscular injection of 0, 0.1, 0.3, or 1 mg uranium/kg (as uranyl acetate, UA) in the presence and absence of stress. Stress treatments were applied for five days prior to injection and ceased just prior to injection. Animals that were stressed had four-fold higher plasma corticosterone levels at the time of uranium exposure (763 vs. 189 ng/ml). Treatment with UA produced time and dose-dependent increases in serum and brain uranium levels, with the highest levels observed on day 3. Exposure to UA decreased ambulatory activity, forelimb grip strength, and weight gain, regardless of stress treatment. Rats treated with 1 mg/kg UA exhibited a 30% decrease in striatal dopamine content 3 days after dosing (59 vs. 41 ng/mg tissue). The effect on dopamine was ameliorated by prior application of stress. No effect of DU or stress was observed on levels of GABA, serotonin, norepinephrine, or GSH in the striatum, hippocampus, cerebellum, or cortex. These results indicate that single exposures to soluble uranium at doses as low as 0.1 mg/kg can have adverse neurological effects. However, uranium also produces renal toxicity (see Tobias et al., this meeting), so it is unclear if the neurological effects are a direct result of uranium or are secondary to renal injury. This work was supported by the US Army Medical Research and Materiel Command DAMD17-1-01-0775.

NEPHROTOXIC EFFECTS OF DEPLETED URANIUM (DU) IN THE RAT. INTERACTIONS WITH STRESS.

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Military use of DU has renewed interest in its toxicology. Data here are part of a larger study to assess neurotoxicology and toxicokinetics of DU and alteration by stress, relating this to nephrotoxicity of soluble DU. To address the issues of the nephrotoxicity of soluble DU in the presence of stress, adult male Sprague-Dawley rats were administered a single im dose of 0, 0.1, 0.3 or 1.0 mg/kg DU (as uranyl acetate). Just before day 0 dosing 1/2 were exposed to swim stress resulting in plasma corticosterone levels of 764+/-131 (n=206) and 190+/-91 (n=200) ng/ml, mean+/-SD, for stressed and unstressed rats, respectively. Sacrifice was on days 1, 3, 7 and 30 (n=4/group/day). DU exposure in all rats resulted in dose-related elevation of blood serum urea nitrogen and creatinine (evident at day 1, further elevated by day 7, elevated with a downward trend by day 30). Serum albumin and hematocrit declined with time in high dose DU rats. Dose dependent increases in serum uranium values peaked at approximately 3 days. Renal lesions included prominent acute tubular necrosis at all treatment levels, primarily noted in the inner cortex and outer medullary stripe, with extension along the medullary rays in mid and high dose treatment groups. Tubular regeneration occurred rapidly in low dose (0.1 mg/kg) rats and was almost complete by day 30. High dose rats had more extensive tubular necrosis and a relatively delayed regenerative response, with regions of multifocal chronic interstitial nephritis and cortical scarring on day 30. Rats receiving the mid dose (0.3 mg/kg) displayed lesions of intermediate severity. Stress had no effect on the nephrotoxicity. These data demonstrate that even very low doses of soluble DU are nephrotoxic, and that stress has no effect on this process. Supported by DAMD 17-1-01-0775, U.S. Army Medical Research and Materiel Command

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PROCEEDINGS, BIOSCIENCE 2004

The Effect of Stress on the Acute Neurotoxicity of the Organophosphate Chlorpyrifos

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Abstract-

The potential of stress to modify the anti-cholinesterase effects of nerve agents is a matter of interest to military and civilian defense planners, since it is a likely component of both combat operations and terrorist attacks. To help address this question, we employed several stress models and assessed their effects on the ability of a neurotoxic organophosphate (the insecticide chlorpyrifos) to inhibit brain acetylcholinesterase (AChE). Stress was developed in male Sprague-Dawley rats over a prolonged period, and was serially measured by body weight, selected neurobehavioral tests and plasma corticosterone concentrations (samples were collected 10 minutes after the stress episode). The stress models (all administered 5 days/week for 6 weeks) were: Group 1 – handling (control); Group 2 – restraint for 30 minutes; Group 3 – four days restraint for 30 minutes, followed by one day swimming for 30 minutes; Group 4 – swimming for 30 minutes (n=8/group). On day 32 (note- the first day of the study is day 0) the rats were bled for blood AChE determination, and on day 39 one-half of the rats in each group (n=4) were administered 60 mg/kg chlorpyrifos subcutaneously. The rats were sacrificed on day 43, and the brains collected for AChE determination. The major stress related findings during the 6-week exposure period included elevated plasma corticosterone in Group 3 (restraint/swim) and Group 4 compared to the controls and Group 2 (restraint) ($p<0.05$). This was noted from day 4 onward. These levels of corticosterone did not differ significantly between Group 3 and Group 4. By day 18, body weight was less in Group 4 than in the other groups. Stress had no effect on blood AChE levels determined on day 32. Chlorpyrifos administered on day 39 significantly ($p<0.05$) inhibited the activity of brain AChE in the control and all 3 stress groups, with no significant difference among them. Thus none of the stress states modified the intracerebral anticholinesterase effect of 60 mg/kg chlorpyrifos in rats.

Key words- stress, corticosterone, organophosphates, acetylcholinesterase, cholinergic toxicity, brain

* presenting author

This was supported by the US Army Medical Research and Materiel Command grants DAMD17-01-1-0775 and DAMD17-99-1-9489.

Introduction-

Chronic stress is known to induce neuronal injury in the brain, especially in the hippocampus (Sapolsky 1996, 1999, 2000). Considerable interest exists as to whether such stress might exacerbate the effects of neurotoxic agents. Relevant to this, both acute and prolonged social stress enhanced the clinical effects associated with degeneration of long myelinated fibers seen in organophosphate-induced delayed neuropathy in chickens (Ehrich and Gross).

Military operations, involving conditions such as unexpected mobilization of reserve units, deployment to a war zone, combat, exposure to casualties and threat of bodily harm, have been correlated with enhanced stress levels (Blood and Kauker; Yerkes). Such operations often include the use of anticholinesterase neurotoxicants as insecticides, and provide potential for exposure to chemical warfare agents with anticholinesterase action. This project, therefore, was undertaken to identify a stress model which might alter such toxicity.

Methods-

Several stress models were induced in rats. These were as follows (all administered 5 days/week for 6 weeks, n=8/group): Group 1- Routine handling (control); Group 2- Restraint- restrained for 30 minutes in a 6 cm diameter x 22 cm length Plexiglas tube (Konarska *et al.*); Group 3- Restraint-swim- 4 days restraint for 30 minutes, followed by 1 day swimming for 30 minutes; Group 4- Swim- swimming for 30 minutes. The schedule of the study is provided in *Table 1.* Periodic in-life measures of stress were determination of body weight, plasma corticosterone levels (samples obtained by orbital sinus bleeding under anesthesia within 10 minutes of cessation of the stress episode) and assessment of motor activity (since no significant change was determined in the latter, this is not further considered in this paper). The schedule for obtaining these data is provided in *Table 1.*

The organophosphate-induced acute neurotoxicity portion of the study was accomplished as follows (also see *Table 1*). Blood was drawn for acetylcholinesterase assay on day 32. A subcutaneous dosage of 60 mg/kg chlorpyrifos was administered on day 39 to n=4/Group. This gave cohorts of chlorpyrifos-dosed and chlorpyrifos non-exposed in each Group (n=4/cohort). The rats were sacrificed on day 43 (CO_2 euthanasia), 3 days after chlorpyrifos administration and 1 day after the last stress episode (*Table 1*). At this time, blood was collected for corticosterone assay and brain for acetylcholinesterase activity determination. For all parts of the study, plasma corticosterone was determined by a radioimmunoassay (Corticosterone 125-I Radioimmunoassssay Kit, ICN Diagnostics, Costa Mesa, CA). Brain and whole blood plasma acetylcholinesterase determination were by the microplate method of Correll and Ehrich. Statistical evaluation of data were as follows. Responses were separately subjected to analysis of variance with mean separation by Bonferroni-corrected multiple comparisons. All calculations were performed using the SAS System (Version 8.02, SAS Institute Inc., Cary, NC 27513).

Results-

Measures of stress included multiple determinations of body weight over the 42-day course of the study. Only the Group 4 (swimming) rats showed an effect, with significant decreases relative to Group 1 (controls) being seen from day 18 on (*Figure 1*). Plasma samples for corticosterone determination were obtained after the last stress for that week and on the day of sacrifice (*Table 1*). These indicated that corticosterone levels were elevated in Group 3, where animals were stressed by restraint for 4 days (Mon-Thurs) and swimming on day 5 (Friday), and in Group 4 (5 days swimming/week) (*Figure 2*). It should be noted that the samples were taken after the single weekly swimming episode for Group 3. Plasma corticosterone levels in Groups 3 (restraint/swim) and 4 (swim) were significantly elevated ($p<0.05$) above Groups 1 (handle) and 2 (restraint) on days 4, 11, 18 and 32, but they were not statistically different from each other (*Figure 2*). Likewise, Groups 1 and 2 did not differ significantly. The elevated plasma corticosterone level as measured on the 5th day of weekly stress continued over the 6-week exposure for Groups 3 and 4.

In order to approximate the levels of corticosterone before and after stress on the first 4 days of the week, animals sacrificed on day 43 (Tuesday, *Table 1*) were divided into 2 subgroups. In each treatment group, 4/8 animals were stressed prior to sacrifice and the remaining 4/8 animals were not so stressed. The results of the corticosterone assays for these animals are shown in *Figure 3*. These findings indicate that elevation of plasma corticosterone seen in Groups 3 and 4 (*Figure 2*) is directly associated with swimming stress, and that such elevations are transient. As an example, Group 4 rats that were swum on day 43 had elevation of plasma corticosterone (*Figure 3*, Group 4, stress). Other members of that group, who had last swum on day 42 (the day before blood samples were obtained, *Table 1*) had normal levels of this hormone on day 43 (*Figure 3*, Group 4, no stress). It appears that post-swimming elevation of this hormone returned to normal within 24 hours. Similarly, Group 3 rats, last swum on day 39, had normal plasma corticosterone levels on day 43, whether or not preterminal restraint was applied (*Figure 3*). Stress in any form did not have an effect on plasma acetylcholinesterase activity, when measured on day 32 of the study (*Table 1*).

For studies of the stress-organophosphate interactions, chlorpyrifos was administered on day 39 (after data for *Figure 1* was collected) to 4/8 animals/group. Sacrifice was on day 43. There was no stress-related alteration of brain chlorpyrifos-induced acetylcholinesterase inhibition (*Figure 4*). In addition, there was no chlorpyrifos associated alteration of plasma corticosterone, whether or not the animals were stressed (*Figures 3, 5*). The data displayed in *Figure 3* represents a combination of chlorpyrifos dosed and not dosed rats to give the simple main effect of presacrifice stress. *Figure 5* demonstrates similar findings when data are reported showing the interactions of chlorpyrifos administration and terminal stress exposure.

Discussion-

Studies concerning the effects of stress on the anticholinesterase-based acute toxicity of organophosphates and carbamates have provided inconsistent data. A major issue relates to the ability of physical stress to alter the permeability of the blood-brain-barrier, enhancing central penetration of previously excluded compounds. The carbamate pyridostigmine bromide is a compound of intense interest, related to its use as a protectant against chemical warfare agents. Friedman, *et al.* have reported that physical (forced swimming) stress disrupted the blood-brain-barrier in mice so that much smaller doses of pyridostigmine bromide were needed to halve the activity of brain acetylcholinesterase. Other studies provide contradictory data, in that single or multiple daily episodes of forced running stress do not change the brain anticholinesterase effects of pyridostigmine bromide in rats (although the multiple episodes enhance the peripheral cholinergic toxicity of that compound [Shaikh, *et al.*]) (Shaikh and Pope; Shaikh, *et al.*).

Organophosphate anticholinesterase compounds have the ability to penetrate the blood-brain-barrier. Short term post-exposure running stress did not alter the cholinergic effects of one such compound (paraoxon) (Shaikh and Pope). The present study extended this work, comparing multiple stress models administered to rats over a 6 week period.

We used intermittent (daily for 30 minutes, 5 days/week) stress over a six-week period, employing restraint, forced swimming or a combination of these two. Using body weight loss and plasma corticosterone elevation as measures, it appears that only forced swimming elicited both weight loss and corticosterone elevation. The latter was also noted in the restraint-swim group, but only if blood was obtained following the single weekly swimming episode. In both groups the swimming-related corticosterone elevation was transient, with levels returning to control levels within 24 hours. None of these long-term intermittent stress paradigms affected the ability of chlorpyrifos to inhibit brain acetylcholinesterase. This is consistent with the findings of Shaikh and Pope using paraoxon after a single forced running episode in rats.

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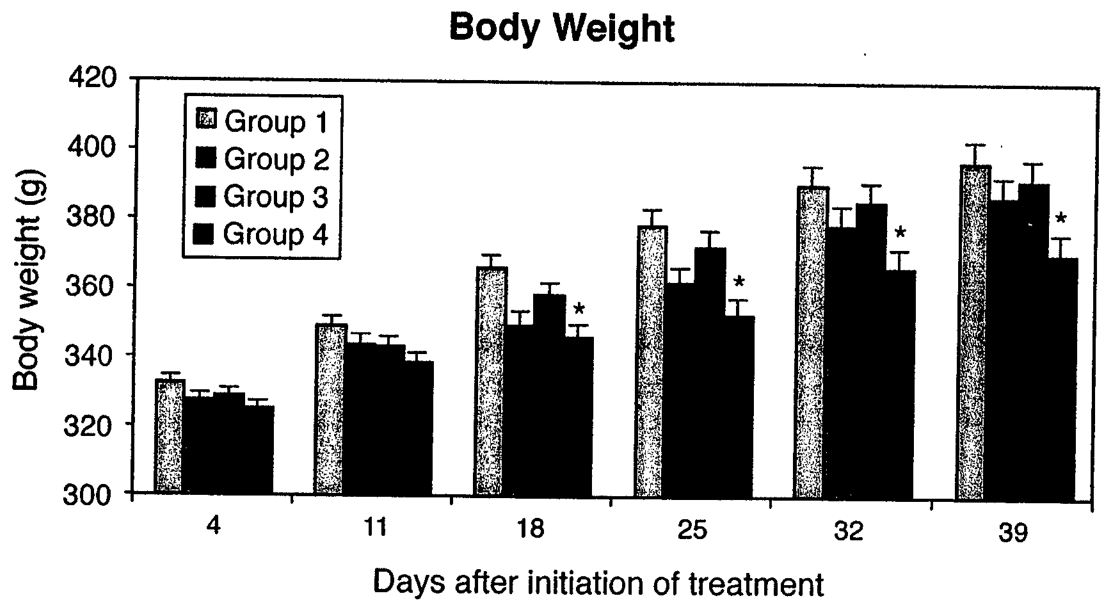

Figure 1. Relative to controls (Group 1) there was significant (*p < 0.05) decrease in body weight of Group 4 (swim) rats from day 18. n=8/group/interval

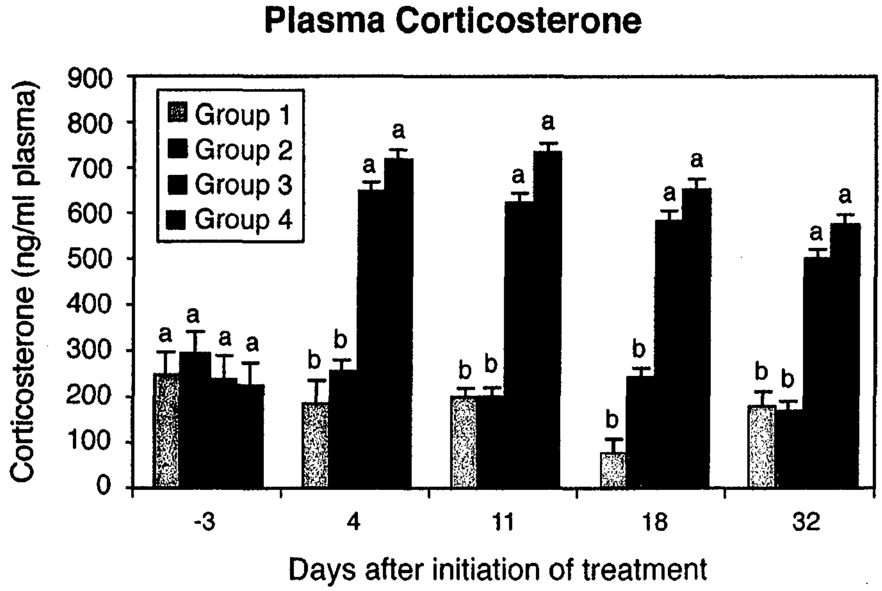

Figure 2. Corticosterone levels of animals by treatment group and study day. The restraint/swim (Group 3) and swim (Group 4) models resulted in elevation of plasma corticosterone on days 4, 11, 18 and 32, a feature not seen in the handling control (Group 1) or restraint (Group 2) animals. Each point represents the mean of 8 observations (± standard error of the mean). Means within a day with no letters in common are significantly different at $\alpha=0.05$.

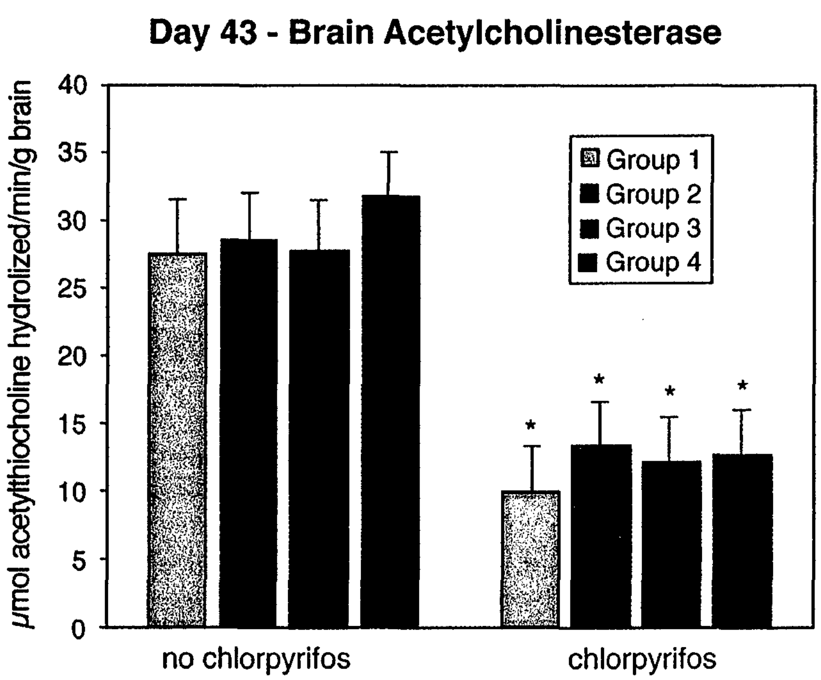

Figure 4. Chlorpyrifos administered on day 39 results in significant (*$p < 0.05$) inhibition of brain acetylcholinesterase on day 43 compared to control (Group 1, no chlorpyrifos) values. There is no effect of any stress paradigm (Group 1- routine handling. Group 2- restraint, Group 3- restraint/swim, Group 4- swim) on this inhibition. n = 4/value.

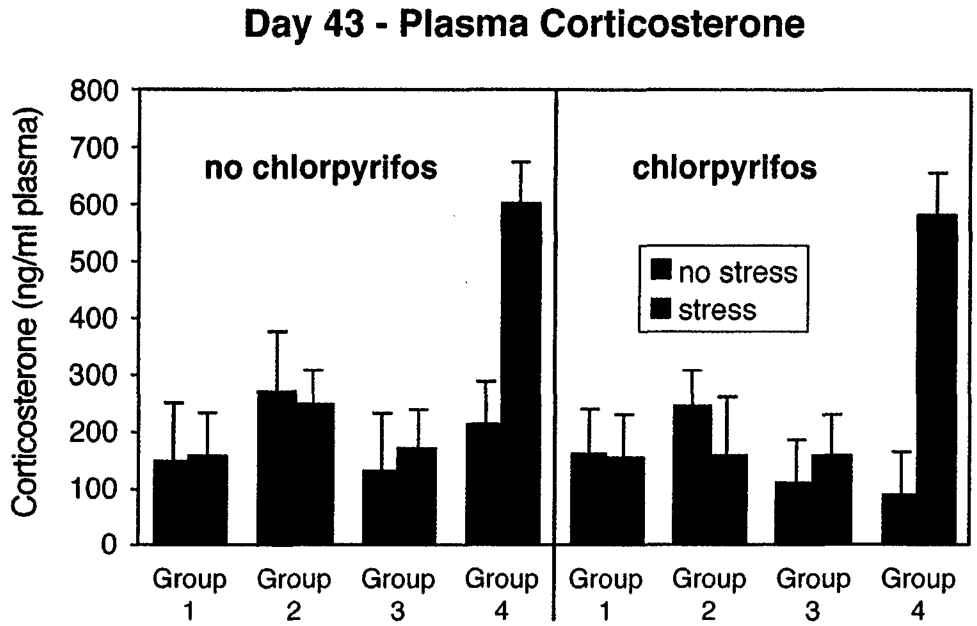

Figure 5. There is no effect of chlorpyrifos on plasma corticosterone levels on day 43 (toxicant administered on day 39) in any stress group (Group 1- routine handling. Group 2- restraint, Group 3- restraint/swim, Group 4- swim). n = 2/value.

2 additional in press manuscripts in PDF format submitted as separate documents. These are –

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THE EFFECT OF STRESS ON THE TEMPORAL AND REGIONAL DISTRIBUTION OF URANIUM IN RAT BRAIN AFTER ACUTE URANYL ACETATE EXPOSURE

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Long-term exposure to depleted uranium (DU) has been shown to increase brain uranium and alter hippocampal function; however, little is known about the short-term kinetics of DU in the brain. To address this issue, temporal and regional distribution of brain uranium was investigated in male Sprague-Dawley rats treated with a single intraperitoneal injection of 1 mg uranium/kg as uranyl acetate. Due to the inherent stress of combat and the potential for stress to alter blood–brain barrier permeability, the impact of forced swim stress on brain uranium distribution was also examined in this model. Uranium in serum, hippocampus, striatum, cerebellum, and frontal cortex was quantified by inductively coupled plasma–mass spectrometry (ICP-MS) at 8 h, 24 h, 7 d, and 30 d after exposure. Uranium entered the brain rapidly and was initially concentrated in hippocampus and striatum. While multiple phases of uranium clearance were observed, overall clearance was relatively slowand the uranium content of hippocampus, cerebellum, and cortex remained elevated for more than 7 d after a single exposure. Prior exposure to stress significantly reduced hippocampal and cerebellar uranium 24 h post-exposure and tended to reduce uranium in all brain regions 7 d after exposure. The application of stress appeared to increase brain uranium clearance, as initial tissue levels were similar in stressed and unstressed rats.

The deposition of uranium in kidney and bone and its subsequent nephrotoxicity has been well documented (Diamond et al., 1989; Leggett, 1989). However, exposure of military personnel to uranium through the use of depleted uranium (DU) munitions has renewed concern about potential health effects of uranium exposure. Recent analysis of urinary uranium levels during deployment in Bosnia demonstrated that deployment increased mean uranium excretion, but not above normal levels (May et al., 2004). Concerns about DU toxicity center on soldiers who were wounded by DU shrapnel and retained fragments of DU. These soldiers excreted elevated levels of uranium

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for at least 10 yr after injury, indicating ongoing mobilization of DU from fragments and the highest rates of uranium excretion were associated with elevated rate of HPRTmutations (McDiarmid et al., 2001a, 2004). Initial health assessment of this group of soldiers reported a positive correlation between elevated uranium excretion and reduced performance on neurobehavioral testing in the absence of renal injury (McDiarmid et al., 2000). These effects later dissipated, but they raised questions about the potential for DU-induced neurotoxicity (McDiarmid et al., 2001b, 2002). Hippocampal slices from rats implanted with DU pellets for 6–12 mo displayed electrophysiological abnormalities characterized by poor excitatory postsynaptic potential/spike (E/S) coupling, also suggesting a neurotoxic effect associated with DU (Pellmar et al., 1999b).

Very little is known about the transport of uranium in the central nervous system. Animal studies demonstrated that brain uranium levels in rats implanted with DU pellets increased during 18 mo after implantation (Pellmar et al., 1999a). This is consistent with previous studies indicating that multiple exposures to soluble uranium compounds via drinking water, injection, and inhalation also increase brain uranium levels (Gilman et al., 1998a, 1998b; Ortega et al., 1989). The International Commission on Radiological Protection (ICRP) has a biokinetic model for uranium, but there are no data on the short-term temporal and spatial distribution of uranium in brain. The need for this data was highlighted in a recent report on kinetics of DU release from solid fragments (Leggett & Pellmar, 2003).

For uranium to enter the brain, it must traverse biological barriers. The blood–brain barrier (BBB) and the blood–cerebrospinal fluid (CSF) barrier are the primary sites of transport of substances from the peripheral circulation into the central nervous system (CNS) and are the major determinants of brain concentrations of polar molecules, such as metals (Zheng, 2001). While controversial, studies have linked acute stress to increased permeability of the BBB (Friedman et al., 1996). Because combat situations are undoubtedly stressful, stress must be considered to accurately depict transport of uranium in the CNS during wartime military operations. This study examined uranium transport in the CNS by measuring uranium in cerebral cortex, hippocampus, striatum, cerebellum, and serum of normal and stressed rats as a function of time following a single intraperitoneal injection of uranyl acetate.

MATERIALS AND METHODS

Materials

Uranyl acetate dihydrate was purchased from Alfa Aesar (Ward Hill, MA). This material contained 0.4% ^{235}U based on ICP analysis. Ultra-trace-metal grade nitric acid (Optima, Fisher) and hydrogen peroxide (Ultrex II, J. T. Baker) were purchased from Fisher Chemical Company (Atlanta, GA). All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO) and were of the highest purity available.

Stress Induction and Uranium Treatment

Five male Sprague-Dawley rats (250–300 g) were used in each group. Stress was induced by five daily episodes of forced swimming. Each 12-min session was composed of swimming for 4 min, resting for 4 min, and swimming for 4 min, as previously described (Friedman et al., 1996). Swimming was conducted in a rectangular tank (19 × 14 in) filled to a depth of 20 cm with room temperature water (20–23°C). Unstressed animals were subjected to the same protocol, but were placed into empty tanks. One hour after the final swimming session, 1 mg/kg uranium was administered as an intraperitoneal injection of uranyl acetate in saline (1 ml/kg). Control rats were injected with 1 ml/kg of sterile saline. After dosing, rats were returned to their home cages and were allowed ad libitum access to food and water. Animals were killed by carbon dioxide inhalation 8 h, 24 h, 7 d, and 30 d following uranium exposure. Saline controls were sampled 24 h after injection.

Corticosterone Determination

Blood was collected from the tail vein using a heparinized syringe immediately prior to dosing with uranium (1 h after last episode of stress). Plasma was prepared by centrifuging blood sample at 1500 × g for 10 min. Corticosterone was determined using the corticosterone ^{125}I double-antibody radioimmunoassay kit (ICN Diagnostics, Aurora, OH) according to manufacturer's instructions. A 100-μl plasma sample was used per tube and samples were run in duplicate.

Sample Preparation and ICP Analysis

Blood samples were collected from the inferior vena cava into serum separator tubes (Becton-Dickinson, Franklin Lakes, NJ). Serum was obtained by centrifugation according to manufacturer's instructions. Brains were removed immediately and frontal cortex, cerebellum, striatum, and hippocampus were isolated by blunt dissection. Samples from both hemispheres were pooled to provide adequate amounts of tissue for analysis. Samples were frozen at –80°C until analysis.

Samples were digested in sealed pressure tubes with 1 ml of concentrated ultra-pure nitric acid at 140°C for 2 h, followed by addition of 0.5 ml of 30% Ultrex II hydrogen peroxide and further digestion at 110°C for 60 min. Samples were then quantitatively diluted to 5 ml with ultrapure water and filtered (0.45 μm) prior to analysis. Uranium analysis was performed by inductively coupled plasma–mass spectrometry (ICP-MS) by the Analytical Section of the Hazard Identification Core in the Southwest Hazardous Waste Program at the University of Arizona (Tucson). Analysis was conducted on an HP 7500a ICP-MS using iridium as an internal standard. Uranium concentration was determined from a standard curve of uranium based on the *m/z* 238 signal. Five repetitions were performed per sample and the average used to calculate uranium concentration. The limit of quantitation of this method was 0.002 ppb.

Recovery was determined from samples spiked with 0.1–10 ppb uranium and determined to be 96–108%.

For Light Microscopy

Kidneys were immersion fixed at sacrifice in 10% neutral buffered formalin; sections were trimmed, embedded in paraffin, and sectioned at 5 μm. Slides were stained with hematoxylin and eosin (H&E) and periodic acid Schiff (PAS) and examined by light microscopy. For the initial examination, slides were read in a blinded (coded) manner such that the uranium dosage status of the rats was not known.

Statistical Analysis

Tissue uranium data was analyzed using a three-way analysis of variance (ANOVA) with treatment, tissue (serum or brain region), and time after dosing as the treatment variables. Where significant differences were found for main effects or interactions, all pairwise comparisons were made using the Student–Newman–Keuls post hoc test; p values $<.05$ were considered to be significant.

To determine if multiple phases of uranium clearance were present, the slopes of the regression lines for each segment of clearance were compared using an unpaired t-test; p values $<.05$ were considered to be significantly different.

RESULTS

Application of five daily episodes of forced swimming produced moderate stress in treated animals. Mean plasma corticosterone levels were two-fold higher in stressed animals (411 ± 17 ng/ml) than in unstressed animals (214 ± 38 ng/ml) at the time of uranium injection. Treatment with a single intraperitoneal injection of 1 mg/kg uranyl acetate did not produce mortality or visible signs of toxicity in stressed or unstressed animals at any point during the experiment.

Statistical analysis of tissue uranium concentrations revealed significant effects of time after administration, tissue, and treatment (control, UA only, UA + stress) as well as all two- and three-way interactions. In saline-treated animals, serum, cerebellum, and cortex contained 0.18 ng U/ml, 0.17 ng U/g, and 0.66 ng U/g, respectively. Significantly higher concentrations were present in hippocampus and striatum at 2.0 and 1.4 ng U/g, respectively. Eight hours after intraperitoneal injection of 1 mg/kg uranyl acetate (UA), uranium concentrations in unstressed animals increased 5- to 100-fold and were significantly higher than corresponding samples in saline-treated controls (Figure 1). Tissue uranium concentrations in stressed animals treated with 1 mg/kg uranyl acetate (UA + stress) were also significantly higher than corresponding control samples but not significantly different than those in unstressed rats. At this time, significant variation in uranium distribution was observed among brain regions in all UA treated rats. The highest uranium levels were observed in hippocampus and striatum, which contained 10.7 ± 2.0 and 18.7 ± 3.3 ng U/g or 3.7×10^{-4}% and 7.4×10^{-4} % of injected dose, respectively.

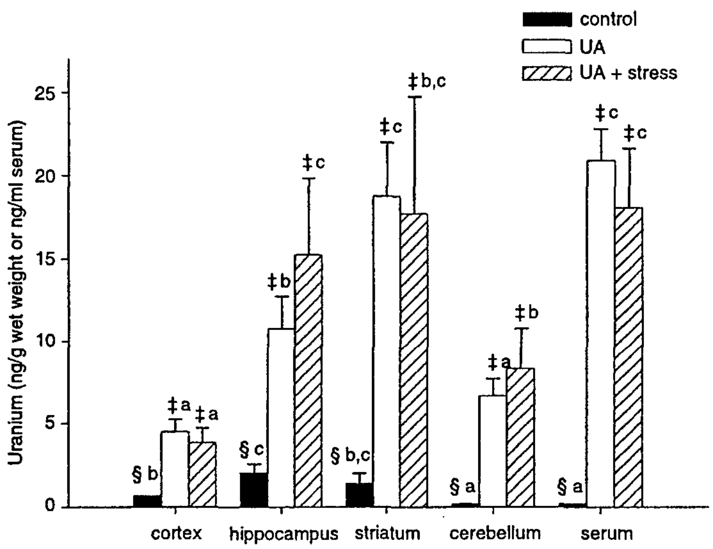

FIGURE 1. Serum and brain uranium concentrations 8 h after injection with 1 mg uranium/kg. Values are presented as mean ± SD ($n = 5$). Within each tissue (i.e., cortex, hippocampus, etc.), treatment groups (i.e., control, UA, UA + stress) that do not share the same symbol (§, ‡, or †) are significantly different ($p < .05$). Columns that do not share the same letter indicate significant differences ($p < .05$) among the five tissues when comparing the same treatment.

Twenty-four hours after UA exposure, uranium concentrations in all samples from stressed and unstressed rats remained significantly greater than corresponding samples in saline treated rats (Figure 2). However, hippocampal and cerebellar uranium levels in stressed animals were significantly lower than corresponding regions in unstressed animals. The uranium content of all tissues except cerebellum in unstressed animals decreased between 8 and 24 h. Surprisingly, cerebellar uranium in unstressed rats was approximately 3-fold higher 24 h after injection than at 8 h (17.8 ± 3.5 ng/g or 1.7×10^{-3}% of injected dose).

Seven days after exposure, uranium levels in hippocampus, cortex, cerebellum, and serum remained significantly higher in unstressed rats than their corresponding controls (Figure 3). At this time, uranium content of all measured brain regions tended to be lower in stressed rats than in corresponding samples from unstressed rats and were not significantly different than control values in hippocampus and striatum. However, serum uranium levels in stressed rats were significantly higher than in unstressed rats and higher than values observed at 24 h. Thirty days after treatment, the uranium contents of all tissues in stressed and unstressed rats were not significantly different than control values (Figure 4). The relatively large error associated with determination

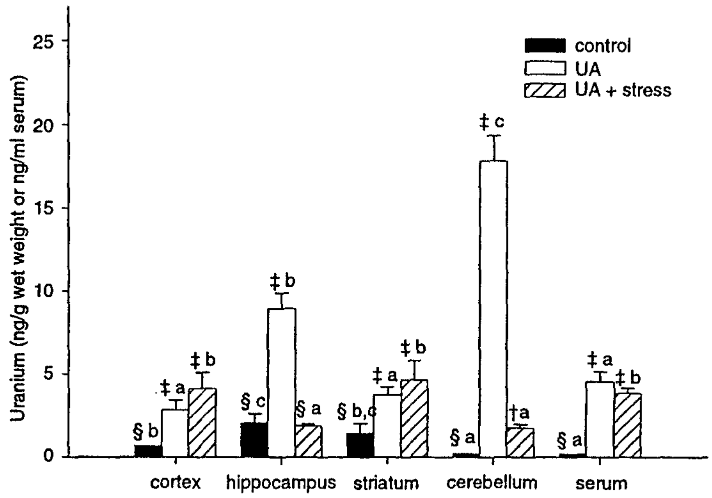

FIGURE 2. Serum and brain uranium concentrations 24 h after injection with 1 mg uranium/kg. Values are presented as mean ± SD (n = 4–5). Within each tissue (i.e., cortex, hippocampus, etc.), treatment groups (i.e., control, UA, UA + stress) that do not share the same symbol (§, ‡, or †) are significantly different ($p < .05$). Columns that do not share the same letter indicate significant differences ($p < .05$) among the five tissues when comparing the same treatment.

of low levels of uranium precluded us from identifying small amounts of retained uranium.

Analysis of uranium in each brain region with respect to time reveals that there are multiple phases of elimination in the measured brain regions, with the terminal phase being much slower than the initial phase (Figure 5). While the number of data points is limited, there also appear to be differences in the rate of uranium clearance from various brain regions.

Kidney Injury

No definitive evidence of tubular necrosis was seen 8 or 24 h after administration of 1 mg/kg depleted uranium, although occasional proteinaceous casts were noted in some animals. Tubular epithelial-cell necrosis and regeneration were seen 7 d postdosing, in 3 of 5 rats. Necrosis (with dystrophic calcification) predominated in one of these and regeneration in the other two. These processes most prominently involved the deeper (juxtamedullary) and outer medullary stripe. In the affected animals, about 20% of the cortex was involved. Proteinaceous tubular casts were present. By 30 d, the cortical and outer medullary stripe tubular epithelial cell population had been largely reconstituted, although foci of active regeneration were still noted. There were no significant changes in the nature or extent of lesions in stressed animals compared to unstressed animals. No lesions were observed in the control rats.

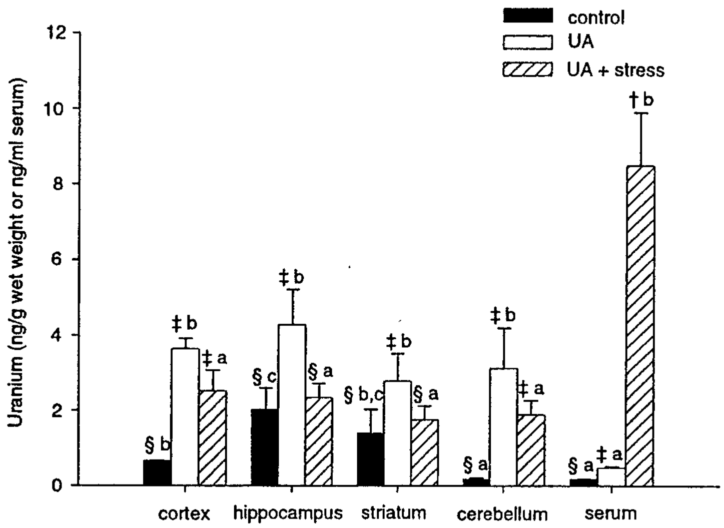

FIGURE 3. Serum and brain uranium concentrations 7 d after injection with 1 mg uranium/kg. Values are presented as mean ± SD (n = 4–5). Within each tissue (i.e., cortex, hippocampus, etc.), treatment groups (i.e., control, UA, UA + stress) that do not share the same symbol (§, ‡, or †) are significantly different ($p < .05$). Columns that do not share the same letter indicate significant differences ($p < .05$) among the five tissues when comparing the same treatment.

DISCUSSION

This study demonstrates that a single peripheral exposure to soluble uranium increases CNS uranium concentrations for at least 7 d in certain brain regions. Peak uranium concentrations occurred less than 8 h after injection in most brain regions, demonstrating that uranium enters the brain rapidly. This is consistent with a recent publication that reported 1000-fold increases in extravascular brain uranium following exposure to 5 μ*M* uranium for 2 min by in situ perfusion (Lemercier et al., 2003). The mechanism by which uranium enters the CNS is unknown, but is likely similar to that for divalent metals since most plasma uranium is present as the divalent uranyl ion ($[UO_2]^{2+}$) (Cooper et al., 1982). Zinc, calcium, manganese, and lead are all transported rapidly from blood into the CNS (Tai et al., 1986; Deane & Bradbury, 1990). Transport rates of calcium and manganese are strongly correlated with plasma concentrations, apparently due to increased transport across the choroid plexus epithelium as plasma concentrations increase (Bouldin & Krigman, 1975; Tai et al., 1986; Takeda et al., 1994; Murphy et al., 1991). If similar mechanisms are involved in uranium transport, plasma concentration will play a key role in determining brain uranium content. Since most military exposure to DU is by

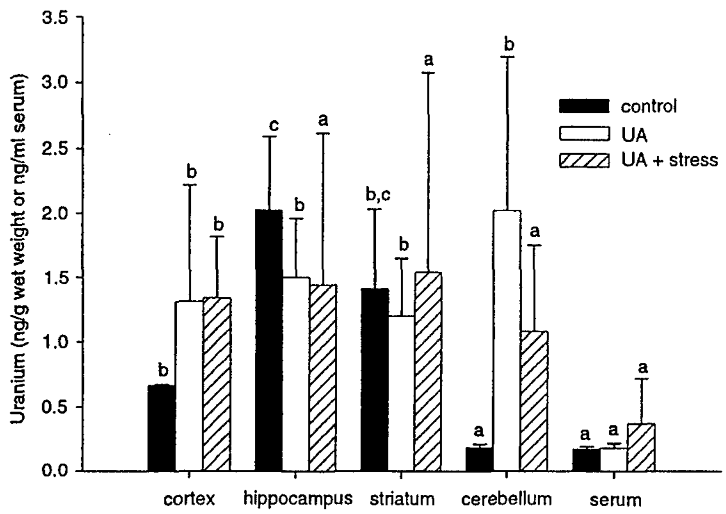

FIGURE 4. Serum and brain uranium concentrations 30 d after injection with 1 mg uranium/kg. Values are presented as mean ± SD ($n = 5$). There were no significant differences in tissue uranium contents among treatment groups (i.e., control, UA, UA + stress). Columns that do not share the same letter indicate significant differences ($p < .05$) among the five tissues when comparing the same treatment.

inhalation of DU oxide particles or embedded DU metal shrapnel, which will produce circulating uranium levels much lower than the current study, studies are currently evaluating CNS uranium levels in rats exposed to lower doses of uranium.

In this study, uranium distributed heterogeneously, with hippocampus and striatum containing the highest levels 8 h after exposure in stressed and unstressed rats, as well as in control (saline treated) samples. The mechanism responsible for the high levels of uranium in these regions is not clear, but preferential accumulation in hippocampal and striatal tissue has been observed with other divalent metals, including zinc, copper, calcium, manganese, and lead (Tai et al., 1986; Roels et al., 1997; Gallez et al., 1998; Sheuhammer & Cherian, 1982; Takeda et al., 1994). High-capacity transport into the cerebrospinal fluid (CSF) leading to accumulation in paraventricular brain regions has been reported for manganese and calcium and may be important for uranium as discussed previously (Murphy et al., 1991; Roels et al., 1997; Tai et al., 1986). Another possibility is the presence of transporters that act on uranium within these tissues such as demonstrated for hippocampal neurons expressing zinc transporter-3 (Frederickson et al., 2000). While the brain regions examined in this study were chosen based on reports of cognition, memory, and motor difficulties in Gulf War veterans, it is possible that brain

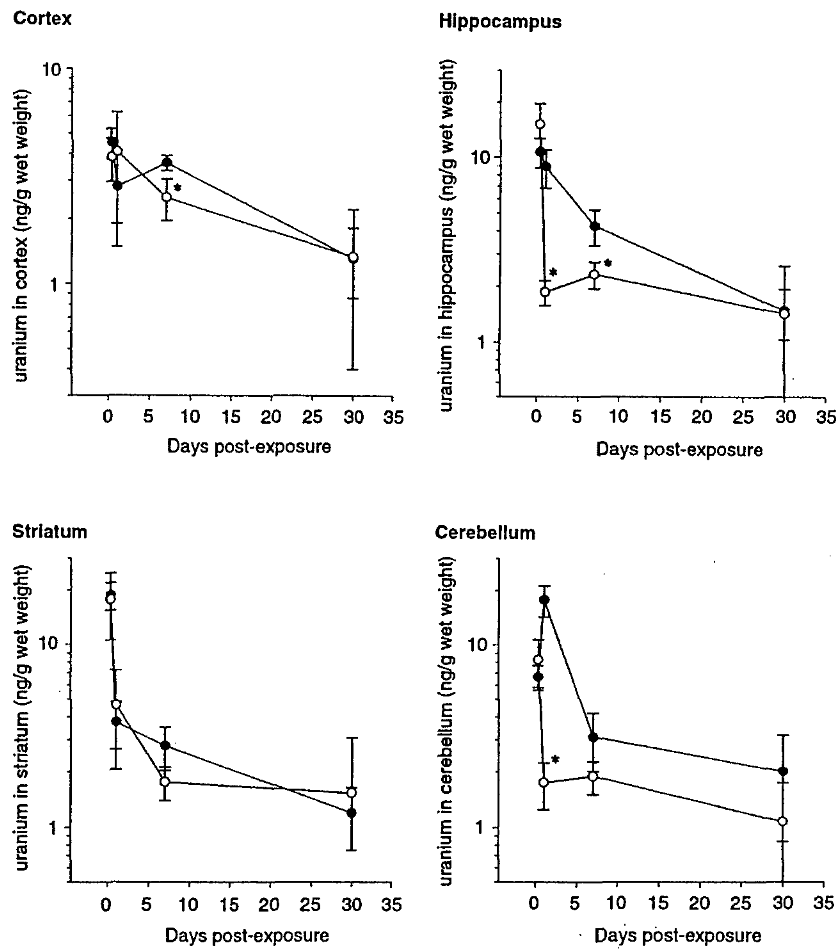

FIGURE 5. Uranium concentrations in brain regions of stressed and unstressed rats at various times after uranium injection. Values are mean ± SD (n = 4–5). Asterisk indicates values in stressed rats that are significantly different from the corresponding time in unstressed rats ($p < .05$). In all regions and treatments, the rate of uranium clearance between d 7 and 30 is significantly slower than the initial phase ($p < .05$).

structures not examined in these studies, such as hypothalamus, may also contain high levels of uranium (Lever & Scheffel, 1998).

More than one phase of uranium clearance was observed in all brain regions (Figure 5). The terminal phase of clearance was relatively slow, and uranium levels in hippocampus, cerebellum, and cortex, but not striatum, remained significantly elevated 7 d after a single exposure. This has previously been observed and a biokinetic model of uranium in adult rats describes three compartments of soft-tissue uranium (ST0, ST1, and ST2) that have clearance half-lives of 2 h, 3 d, and 100 d, respectively (Leggett & Pellmar, 2003). In the only other study of regional brain uranium levels, Pellmar et al. (1999a) reported uneven distribution of brain uranium 18 mo after implantation of DU pellets. In that study, dose-dependent increases in tissue uranium were observed in frontal cortex and cerebellum with cerebellar levels being the highest (Pellmar et al., 1999a). The relatively long retention of uranium in cerebellum and cortex observed in the present study may account for accumulation of uranium in these regions following multiple and chronic exposures such as seen in the Pellmar study. From the present study, it is not clear why hippocampal uranium levels did not increase during long-term exposure to implanted DU (Pellmar et al., 1999a), but there may have been differences in brain distribution due to the relatively low plasma uranium levels in the long-term study.

Despite prior reports demonstrating that acute stress can increase permeability of the BBB (Friedman et al., 1996), forced swim stress did not increase uranium levels in any brain region. Our findings agree with other studies demonstrating that acute stress does not increase (Kant et al., 2001; Grauer et al., 2000) and may actually reduce BBB permeability (Sinton et al., 2000). It should be noted, however, that the forced swimming model used in this study was a moderate stressor that only increased circulating corticosterone levels twofold at the time of uranium exposure. The effect of stress on BBB permeability is not completely understood, but may be related to circulating levels of corticosterone, and it is possible that more severe stressors would produce different effects.

Stress was not without effect, as forced swimming prior to uranium exposure significantly reduced uranium levels in hippocampus and cerebellum 24 h after administration (Figures 2 and 5) and tended to reduce uranium concentrations in all brain regions at 7 d (Figure 3). Forced swimming also resulted in marked increases in plasma uranium 7 d after exposure (Figure 3). This phenomenon is not unique to uranium as previous studies demonstrated that stress alters trace metal distribution and clearance. For example, brain zinc levels are reported to decrease by 30% following cold-restraint stress, while stress increases serum and urinary zinc levels (Spencer et al., 1985; Itoh et al., 1993; Izgut-Uysal et al., 2000). In a similar manner, stress might alter the subcellular distribution of uranium such that more metal is in a readily exchangeable state, resulting in decreased brain uranium and increased serum uranium. While stress appears to reduce the brain burden of uranium, the combined effects of stress and uranium on brain function remains unclear.

The presence of uremia has been shown to alter the permeability of the BBB (Fishman, 1970). Kidney function was not determined, but treatment with 1mg/kg uranium produced no definitive tubular degeneration during the first 24 h after exposure and uremic conditions were unlikely to be present. At this time, uranium was already decreasing in most brain regions, so while mild uremia may have occurred at later times, it is unlikely that uremia affected initial CNS uptake of uranium.

In this study, brains were not perfused prior to uranium analysis so uranium in blood influenced tissue uranium concentrations. In the brain regions studied, blood volumes are reported to be between 1.5 and 6.5 ml/100 g tissue (Pullen et al., 1991; Tai et al., 1986). Due to the small volumes of blood in these brain regions and the relatively low levels of uranium in serum at time points longer than 8 h, it is unlikely that uranium in the vasculature contributed significantly to tissue uranium values. An upper bound estimate of the vascular uranium contribution to measured tissue uranium at 24 h is 0.3 ng uranium/g tissue (assuming 6.5 ml blood/g tissue).

In conclusion, rapid transport of uranium from the periphery into the central nervous system occurs. Once in the brain, uranium exhibits heterogeneous distribution among brain regions and is retained by some regions for greater than 7 d, though clearance was affected by stress. These results raise questions regarding the species of uranium that enter the brain, the transport pathway used by uranium at various plasma concentrations, and the cellular and subcellular distribution of uranium in the brain of normal and stressed animals. While the nervous system is not traditionally considered a target for uranium toxicity, this study emphasizes the need for further evaluation of uranium neurotoxicity.

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Heat Shock Proteins and Acquired Resistance to Uranium Nephrotoxicity

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Abstract:

Previous studies have demonstrated that prior exposures to uranium can produce acquired resistance to uranium nephrotoxicity. In this study, the potential role for heat shock proteins (Hsps) in acquired resistance to uranium nephrotoxicity was explored. Pretreatment of male Sprague Dawley rats with a conditioning dose of uranyl acetate (5 mg/kg, i.p.) was found to diminish the severity of proximal convoluted tubule necrosis and azotemia produced by a subsequent, higher uranyl acetate dose (10 mg/kg, i.p., 10 days after the conditioning dose). Kidney homogenates from rats euthanized at the end of the conditioning period were found to contain elevated levels of Hsp25, Hsp32, and Hsp70i, but not Hsc70. Immunochemical staining of renal sections for Hsp25 and Hsp70i revealed that these proteins were prominently expressed in tubular epithelial cells in uranyl acetate pretreated animals. Morphological characteristics and staining for proliferating cell nuclear antigen (PCNA) indicated that the cells expressing high levels of Hsps were regenerating. In RK3E and LLC-PK1 renal epithelial cells in culture, Hsp induction by thermal pretreatment did not afford protection from uranyl acetate cytotoxicity. Further, treatment of RK3E and LLC-PK1 cells with uranyl acetate did not result in induction of Hsps, as occurs with other nephrotoxic heavy metals. These observations suggest that while stress proteins are elevated in acquired resistance to uranyl acetate *in vivo*, they are not responsible for diminished uranium nephrotoxicity but are an epiphenomenon of tubular epithelial regeneration.

Keywords: Uranium; Uranyl Acetate; Heat Shock Proteins; Nephrotoxicity

1. Introduction

The kidney is the major target organ for uranium toxicity. Both inhalation and ingestion of uranium compounds have been shown to produce renal injury in laboratory animals characterized by damage to the glomerulus and proximal tubular epithelium (Avasthi et al., 1980; Haley, 1982; Diamond et al., 1989; Leggett, 1989). Experiments in animal models have demonstrated that exposure to toxic, but non-lethal, doses of uranium can reduce the renal toxicity of subsequent doses in a process termed *acquired resistance* (MacNider, 1929; Honda and Sudo, 1987). The phenomenon of acquired resistance is not unique to uranium and has been described for other renal toxicants, including mercury and gentamicin, as well as for acute injury from ischemia (Zager et al., 1994; Elliott et al., 1982; Goering et al., 2000). The mechanism responsible for acquired resistance is unknown, but altered hemodynamics and glomerular filtration, decreased absorption of toxicant, soluble factors present during uremia, induction of free radical scavengers, and induction of heat shock proteins (Hsps) have been proposed to explain this phenomenon (Honda et al., 1987; Hishida et al., 1991).

Hsps, particularly Hsp25 and Hsp70i are associated with cytoprotection in a variety of models (Ciocca, 1992; Salminen et al., 1997). Evidence for a role of Hsp induction in uranium acquired resistance is provided by the study of Mizuno et al. (1997). They found that induction of acquired resistance from a conditioning dose of uranyl acetate (5 mg/kg) in the rat was associated with increased expression of Hsp73[1] in kidney. Four weeks after the conditioning dose, Hsp73 levels returned to normal and the acquired resistance from the conditioning dose was lost. A lower dose of uranyl acetate (2 mg/kg) inadequate to produce acquired resistance did not increase Hsp73 expression. Additional support for a role of Hsp induction in acquired resistance is provided by experiments with LLC-PK_1 cells. Subjecting the cells to thermal stress, the classical inducer of Hsps, was shown to lead to resistance to uranyl acetate cytotoxicity (Furuya et al., 1997). Although Hsps were not measured directly in this study, the observations are nonetheless consistent with protection from Hsp upregulation.

[1] Hsp73 was used to identify the constitutive member of the Hsp70 family of proteins. For the purposes of the study presented here, Hsc70 is used to designate this protein.

While this evidence is suggestive that Hsp induction is responsible for uranium acquired resistance, additional testing of this hypothesis is warranted. If increased Hsp levels account for the protection, it should be possible to demonstrate induction in the specific renal cell types that are targets for uranium toxicity, principally the tubular epithelium. No experiments of this type have yet been reported. Also, while the observations of Mizuno and coworkers suggest involvement of Hsp73 [Hsc70], there has been little in the way of examination of other Hsps that might be upregulated during a conditioning dose of uranyl acetate. Consequently, a role for other Hsps in acquired resistance cannot be ruled out.

The objective of the study reported here was to extend the previous work of others by examining the effect of a conditioning dose of uranyl acetate in rats on a set of Hsps, including those most commonly associated with upregulation by toxicants and cytoprotection. Immunohistochemical staining of uranyl acetate-treated rat kidneys was used to determine whether Hsp upregulation occurred specifically in cells most susceptible to uranium, the tubular epithelium. Cell culture studies were used to verify that uranyl acetate is capable of inducing Hsps *in vitro*, and that elevated Hsps are associated with protection from uranium cytotoxicity.

2. Material and methods

2.1 Materials.

Uranyl acetate (>98% purity, Alfa Aesar, Ward Hill, MA) was dissolved in sterile saline for injections. Bovine serum albumin (BSA), phenylmethylsulfonyl fluoride (PMSF), Tris-base, sodium dodecyl sulfate (SDS), citric acid, calcium chloride, cell culture antibiotics (penicillin, streptomycin, and amphotericin B), glycine, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide disodium salt (XTT), Tween-20, and dithiothreitol were obtained from Sigma Chemical Co. (St. Louis, MO). ACS grade methanol, xylene, formaldehyde, sodium chloride and normal saline (0.9%) were obtained from Fisher Scientific (Pittsburg, PA).

2.2 Animals and treatments.

Male Sprague-Dawley rats (250-275 g, Harlan Sprague-Dawley, Indianapolis, IN) were acclimated to standard housing conditions (12 hour day/night cycle; *ad libitum* Purina rodent chow and water) for one week prior to experimentation. Animals were maintained in accordance with the procedures outlined in the *Guide for the Care and Use of Laboratory Animals* (National Academy Press, Washington, D.C., 1996) under an animal protocol approved by the Institutional Animal Care and Use Committee. Rats were divided into three groups: Control rats (n=10) were treated with normal saline (1 ml/kg body weight, i.p.) on Day 0 and Day 10; Unconditioned rats (n=13) were treated with normal saline on day 0 and 10 mg/kg uranyl acetate, i.p., on Day 10; and Conditioned rats (n=13) were treated with 5 mg/kg uranyl acetate, i.p., on Day 0 and 10 mg/kg uranyl acetate, i.p., on Day 10. The size and timing of doses were based on a previous study that examined acquired resistance to uranyl acetate (Mizuno et al., 1997). On Day 10, three animals each from the Control, Unconditioned and Conditioned groups were euthanized, and kidney and liver tissues were harvested for Western blot and immunohistochemical analysis. On Day 15, remaining animals were euthanized by CO_2 asphyxiation. Blood was drawn from the inferior vena cava into serum collection tubes (BD Vacutainer, Franklin Lakes, NJ) immediately after euthanasia and serum was separated by centrifugation. Serum was stored at -20° C until analyzed for blood urea nitrogen (BUN) and creatinine concentrations. Portions of the kidney and liver were taken for histopathological examination and Western blot analysis.

For acute uranium exposures, naïve rats were treated with a single dose of 10 mg/kg uranyl acetate, i.p. in normal saline. Rats receiving an equivalent volume of normal saline (1 ml/kg, i.p.) alone served as controls. Twenty-four hours after treatment, Hsp induction and renal histology were evaluated as described below.

2.3 Serum Chemistry.

Serum was analyzed in triplicate for blood urea nitrogen (BUN) by measuring the loss of NADH coupled to ammonia production from urea using a commercially available kit (Infinity BUN reagent, Cat# 63-100P, Sigma Chemical Co., St. Louis, MO). Serum creatinine was measured in duplicate on a Hitachi 911 Serum Chemistry Analyzer using a clinical reagent kit according to the manufacturer's directions (Diagnostics Specialty, Costa Mesa CA).

2.4 Histopathology.

Immediately after euthanasia, a four-millimeter transverse section from each kidney was placed in a tissue cassette and fixed in 10% neutral buffered formalin for three hours. The tissues were rinsed and stored in 95% ethanol until being processed routinely and embedded in paraffin. Three sequential sections 4-6 µm thick were cut from the same block to facilitate comparison of the localization of Hsp induction with kidney damage. One section was stained with hematoxylin and eosin (H&E) for histopathologic evaluation, one was immunostained for Hsp25, and the third was immunostained for Hsp70i. Histopathological changes were evaluated by light microscopy and scored by a pathologist in a blinded fashion. Analysis focused on proximal tubular epithelium due to the sensitivity of this region of the tubule to uranium toxicity (Haley et al., 1982). Proximal tubules were identified as eosinophilic cuboidal epithelium with centrally located nuclei. Distal tubules were identified as thinner basophilic cuboidal epithelium with apical nuclei. The severity of the tubular injury was recorded using a grading scale of 1 to 5, related to the extent and severity of the tubular involvement; 1 (normal), 2 (mildly affected), 3 (moderately affected, but sparing tubular epithelium in most tubules), 4 (moderately affected, with loss of tubular epithelium), and 5 (severely affected). Undamaged and regenerating epithelial cells were not differentiated for scoring purposes.

2.5 Western Blotting.

Four hundred milligrams of kidney were homogenized in sample buffer (0.05 M Tris[hydroxymethyl]aminomethane (Tris-base), 2% sodium dodecyl sulfate, 10 mM dithiothreitol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, pH 6.8), boiled for five minutes and stored at -80° C. Twenty micrograms of protein from each sample were resolved by electrophoresis on a 12.5% acrylamide gel (Laemmli, 1970) and blotted to a Hybond-ECL Western membrane (Amersham, England) using a semi-dry blotting apparatus (Millipore, Bedford, MA) with one-half strength Towbin buffer (10% methanol, 96 mM glycine, and 10 mM Tris-base). Membranes were blocked with TBS (20 mM Tris-base, 500 mM sodium chloride, pH 7.5) containing 3% non-fat dry milk and probed with one of the following primary antibodies obtained from Stressgen (Victoria, B.C., Canada): anti-Hsp25 (SPA-801;

rabbit polyclonal), anti-Hsp32 (SPA-895; rabbit polyclonal), anti-Hsc70 (SPA-816 rabbit polyclonal), or anti-Hsp70i (SPA-810; mouse monoclonal). All antibodies were diluted 1:1000 in TTBS (TBS with 0.05% Tween-20) containing 1% non-fat dry milk and incubated with membranes for 18 hours at 24° C with continuous shaking. Alkaline phosphatase-conjugated sheep anti-mouse or donkey anti-rabbit secondary antibodies (Amersham, England), as appropriate, were diluted 1:3000 in TTBS containing 1% non-fat dry milk and incubated with the membrane for one hour at 24° C with continuous shaking. After washing the membrane in TTBS, the alkaline phosphatase colorimetric substrate BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) was added to provide a permanent, visual signal of primary antibody binding. For Hsc70, chemiluminescence detection was performed using a goat anti-rabbit secondary antibody conjugated to horseradish peroxidase and luminescent substrate (Santa Cruz Biotechnologies, Santa Cruz, CA). Luminescence was detected digitally with a Fluor-S imager (Bio-Rad Laboratories, Richmond, CA). Relative Hsp protein levels were quantified by densitometry (SCION image, Scion Corp, Fredrick, MD).

2.6 Immunohistochemical detection of Hsp25 and Hsp70.

For immunostaining, sections were deparaffinized with xylene and rehydrated by immersion in 100% ethanol two times for one minute each, 95% ethanol for one minute, and double distilled water (ddH_2O) two times for two minutes each. Endogenous phosphatase activity was quenched by submerging the slides in 1 M citric acid for ten minutes. The slides were then washed in ddH_2O three times for two minutes each. To help recover epitopes masked by the formalin fixation, the sections were incubated in a solution of 0.1% trypsin (from porcine pancreas, Sigma Chemical Co., St. Louis, MO), 20 mM Tris-base, 0.1% calcium chloride, pH 8.0 for twenty minutes at 24° C. Sections were blocked by incubation with 25% normal goat serum in TBS containing 3% BSA at 37° C for one hour. Preliminary experiments (not shown) demonstrated that this solution prevented non-specific binding of immunoglobulins to necrotic areas. Anti-Hsp25 or anti-Hsp70i antibody (SPA-801 or SPA-810, Stressgen, B.C., Canada) was diluted 1:200 in blocking solution and incubated with the sections at 37° C for one hour and then at 24° C for 18 hours. The sections were washed three times in TBS for two minutes each. Biotinylated secondary antibodies (Southern

Biotechnology Associates, Inc., Birmingham, AL) were diluted 1:500 in TBS containing 3% BSA, placed on the slides and incubated at 24° C for 30 minutes. Streptavidin-linked alkaline phosphatase (KPL, Inc., Gaithersburg, MD) was diluted 1:200 in TBS containing 3% BSA, placed on the slides and incubated at 24° C for 30 minutes. Primary antibody binding was visualized with the Histomark Red detection kit (KPL, Inc., Gaithersburg, MD) producing a dark red stain indicating Hsp localization. The Hsp32 antibody was not amenable to immunohistochemical analysis.

2.7 Immunohistochemical detection of Proliferating Cell Nuclear Antigen (PCNA)

Paraffin sections were deparaffinized with xylene (3 x 5 minutes) and rehydrated by immersion in 100% ethanol, 95% ethanol, 70% ethanol, 50% ethanol and finally distilled water for 5 minutes each. Endogenous peroxidase activity was quenched by immersion in 0.2% hydrogen peroxide for 5 minutes. Sections were blocked with 1.5% normal goat serum in PBS for 1 hour at room temperature. A monoclonal anti-PCNA primary antibody (Santa Cruz Biotechnology) was diluted 1:100 in blocking solution and incubated with sections for one hour at room temperature. Detection was performed with biotinylated goat anti-mouse IgG secondary antibody and streptavidin-biotin-HRP conjugated tertiary reagent followed by incubation with DAB substrate (Santa Cruz Biotechnology, ABC detection kit). Slides were counterstained briefly with hematoxylin. The number of S phase cells was determined by counting the number of nuclei staining strongly for PCNA in a 200X field. Three fields were counted per section for each animal.

2.8 Apoptosis detection by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL).

The TUNEL assay was performed to detect apoptotic cell death. Kidney sections were heated at 55° C for 30 minutes, then deparaffinized in xylene and rehydrated using a descending ethanol series. A positive control slide was incubated with DNAse I (500 μg/ml, Roche Diagnostics, Indianapolis, IN) for 10 minutes at room temperature in order to induce DNA strand breaks. The TUNEL staining was done in accordance with the manufacturer's instructions using modifications to Portera-Cailliau's method (Portera-Cailliau et al., 1994). Diaminobenzidine (Sigma Chemical Co., St. Louis, MO) was used as the detection agent

(Ornberg, 2001). Slides were coded and microscopic counts of TUNEL-positive tubular epithelial cells were made at 200X magnification in a blinded fashion. Fifteen fields per slide were assessed. Counts are reported as mean TUNEL-positive stained cells per slide ± SD (n=3 animals per group).

2.9 Cell culture and toxicity determination.

RK3E rat kidney epithelial cells and LLC-PK1 porcine renal epithelial cells were purchased from the American Type Culture Collection (Rockville, MD, CRL-1895). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Sigma Chemical Co., St. Louis, MO) containing 4.5 g/L glucose, 10% fetal bovine serum, 100 U/ml penicillin and 100 mcg/ml streptomycin. To examine stress protein response, cells were grown to near confluency on 35-mm dishes and subjected to sub-lethal heat stress or treated with up to 1 mM uranyl acetate. Cells were heat stressed by floating sealed culture dishes in a water bath to achieve a temperature in the culture medium of 42° C for 30 minutes. The media was changed immediately after the heat stress. Cells were harvested 24 hours after uranyl acetate treatment or heat stress by scraping into Western blot sample buffer (described above) and Hsp induction was evaluated by Western blotting (see above). To evaluate the ability of Hsp induction to reduce uranium toxicity, cells were plated in 96-well plates at 3 x 10^3 cells/well. Cells were grown overnight, subjected to sublethal heat stress as described above, and treated 24 hours later with varying concentrations of uranyl acetate. Previous studies indicate that similar treatment regime produce maximal Hsp induction in renal epithelial cells between 12 and 24 hours (Meldrum et al., 2001; Wang et al. 1996). Toxicity was evaluated 24 hours after uranyl acetate treatment by the XTT assay (Scudiero et al., 1988). XTT was added to the culture medium, and viability was determined four hours later by optical density (450nm) measurements (Divine et al., 1999). Each experiment was conducted on three separate occasions.

The effect of prior exposure to uranyl acetate on subsequent uranium toxicity was examined in LLC-PK1 cells as previously described (Furuya et al., 1997). LLC-PK1 cells were grown to confluency on 35mm dishes. Cells were treated with various concentrations of UA in serum-free growth media for 24 hours. After treatment, cells were washed to remove UA, removed from dishes with trypsin and replated on new 35mm dishes at 2 x 10^5 cells per dish. Cells were allowed to grow to confluency (`6 days) and were again treated with UA in serum-free media. Cell viability was determined by measuring LDH release 48 hours after second UA treatment.

2.10 Protein Determination.

Protein was measured with the Micro Protein Determination assay (Sigma Chemical Co., St. Louis, MO) using BSA as standard.

2.11 Statistics.

Plasma BUN and serum creatinine data are expressed as means ± SE values and were analyzed using a one-way ANOVA, followed by the Newman-Keuls post-hoc test. Histopathology scores were compared using the non-parametric Wilcoxon Rank Sum test. Cytotoxicity data from cell culture experiments are expressed as percent of control and were analyzed by one-way ANOVA followed by the Tukey's post-hoc test for all pair-wise comparisons. Differences were considered significant when $p<0.05$.

3. Results

3.1 Histopathology, renal function, and Hsp induction in a model of acquired resistance

Male Sprague-Dawley rats treated with a conditioning dose of uranyl acetate (5 mg/kg, i.p.) exhibited diminished weight gain during the subsequent 10 days. Animals receiving the conditioning uranyl acetate dose gained an average of 12.2 g in this period compared to a gain of 39.8 g in saline treated animals.

Kidney sections taken from rats 10 days after a conditioning uranyl acetate dose showed moderate proximal tubular necrosis and tubular changes consistent with regeneration from proximal tubular injury (Figure 1A and 1B). Tubules were slightly dilated with flattened basophilic epithelial cells and hyperchromatic nuclei; scattered lumina contained eosinophilic debris. There was no apparent interstitial edema, mononuclear cell infiltration or glomerular changes in conditioned rats. Renal sections from saline-treated controls showed normal renal morphology.

Conditioning with uranyl acetate significantly increased the number of proliferating cells in kidney sections stained for PCNA. Sections from UA conditioned rats contained 130 $\pm$ 44 PCNA positive nuclei/field versus 7 $\pm$ 2 in control animals. Most cells that stained positively for PCNA expression exhibited the flattened morphology typical of regenerating cells, however some PCNA positive cells appeared more fully differentiated (Figure 1C and 1D).

Conditioning with uranyl acetate increased levels of stress proteins in kidney homogenates. In conditioned animals, Hsp25, Hsp32, and Hsp70i were increased 4.6-, 1.7-, and 7.4-fold, respectively (Figure 2). In contrast, levels of the 70 kDa constitutive heat shock protein (Hsc70) were comparable between the control and treated groups (Figure 2). To determine if stress protein induction from uranyl acetate was specific to kidney, Hsp expression was also evaluated in liver, which is not a principal target organ for uranium toxicity. Uranyl acetate treatment had no effect on hepatic levels of these Hsps (data not shown).

Localization of the stress protein response within kidney tissue to a conditioning dose of uranyl acetate was evaluated by immunohistochemical staining. Expression of Hsp25 and Hsp70i was only weakly detectable immunohistochemically in the tubular epithelium of control rats (Figures 3A & 3C). In the uranyl acetate-conditioned rats, however, Hsp25 was prominently expressed in tubular epithelial cells that exhibited morphological changes suggestive of active regeneration (Figure 3B). Hsp70i was visible in most cell types but was also concentrated in regenerating tubular epithelium (Figure 3D).

To confirm that conditioning reduced the toxicity of a subsequent uranyl acetate dose, nephrotoxicity was compared in conditioned and unconditioned rats treated with 10 mg/kg (i.p.) uranyl acetate. Five days after treatment, unconditioned rats exhibited impaired renal function with marked azotemia (Table 1). Histologically, extensive necrosis of proximal tubular epithelium was present in the cortex of unconditioned rats (Figure 4). In some areas, necrosis and sloughing of tubular epithelium was complete, leaving denuded basement membrane. Tubular necrosis extended to the outer stripe of the medulla. Cortical involvement was prominent, and the outer stripe was more extensively affected. Significantly less damage to the tubular epithelium occurred in conditioned rats (Figure 4, Table 1). In conditioned rats, the resistant epithelium appeared to be largely derived from regenerating cells characterized by a flattened, basophilic morphology. A non-statistically significant but positive protective effect of conditioning was also apparent in assays of renal function, as prior exposure to uranyl acetate reduced the azotemia associated with a second exposure to uranyl acetate (Table 1).

Although a protective effect from conditioning was observed overall, there was considerable variability among animals. Conditioned animals could be divided into two groups with respect to diminished toxicity - "responders" and "non-responders". Non-responders to the conditioning uranyl acetate dose (n = 5 out of 9 in the Conditioned group) gained more than 2 g/day in body weight (total of 22 to 44 g) after the conditioning dose, which was similar to control rats (24 to 55 g). In contrast, the responder animals showed diminished weight gain (less than 2 g/day) during this time frame (total of -32 to 14 g). Five days after the challenge dose of 10 mg/kg uranyl acetate, BUN and creatinine levels were significantly lower in conditioned responders (150 ± 30 and 170 ± 34 mg/dL, respectively) than non-responders (287 ± 163 and 556 ± 76 mg/dL) and unconditioned animals (283 ± 38 and 498 ± 45 mg/dL). BUN and creatinine concentrations in non-responding, conditioned rats were not significantly different from values in unconditioned animals. Similar results were observed for histology scores, with responders being significantly lower than non-responders and unconditioned animals (median 2, 3, and 4, respectively).

3.2 In vitro protection by Hsp induction and UA pretreatment

To determine if elevated levels of Hsps are associated with protection from uranium *in vitro*, uranyl acetate toxicity was examined in control and heat stressed RK3E rat kidney epithelial cells and LLC-PK1 porcine kidney epithelial cells. RK3E and LLC-PK1 cells heated to 42° C for 30 minutes had increased levels of Hsps 24 hours after heat stress (Figure 5B and 5C). However, when thermally pre-treated cells were treated with uranyl acetate 24 hours after heat stress, uranyl acetate cytotoxicity was not altered (Figure 6).

Prior exposure of LLC-PK1 cells to 1mM UA significantly reduced toxicity of a subsequent exposure to UA (Figure 7). However, Hsp expression was no different than control cells (Figure 5C).

3.3 Hsp induction by uranyl acetate relative to tissue injury after acute exposure

Exposure to most nephrotoxic heavy metals results in rapid Hsp induction that precedes renal injury (Goering et al., 2000; Somji et al., 1999). To determine if uranium produced Hsp induction in the same time frame as other nephrotoxic metals, Hsp induction and toxicity were examined in rats, RK3E and LLC-PK1 cells following acute exposure to uranyl acetate. Twenty-four hours after treatment, there was histological evidence of renal injury in rats treated with 10 mg/kg uranyl acetate. Nephrotoxicity was manifest by scattered degenerative changes in the proximal tubular epithelium in the outer stripe of the medulla and proximal tubules of the cortex. Necrotic cells were characterized by pyknotic nuclei, increased eosinophilic staining of the cytoplasm, and were often sloughed into the lumen. Apoptotic cells were also present in kidneys of treated animals. In treated animals, 39.0 ± 8.7 TUNEL positive cells were present per microscopic field, which was significantly higher than the number of apoptotic cells in kidneys sections from control animals (0.17 ± 0.39, $p< 0.05$). However, no increases in BUN were evident at this point (data not shown), suggesting that gross renal function had not yet been compromised. Despite considerable damage to the tissue, there were no detectable differences in Hsp25, Hsp32, Hsc70 or Hsp70i levels between control and treated animals (Figure 2). In similar studies with RK3E cells, cell viability was reduced by 50% 24 hours after treatment with 750 μM uranyl acetate. However, there was no detectable induction of Hsp25 or Hsp70i at this time in cells treated with up to 1 mM uranyl acetate (Figure 5B). Similarly, no induction of Hsps occurred in LLC-PK1 cells treated with up to 1mM UA for 24 hours (Figure 5C).

4. Discussion

The ability of pretreatment with uranium to reduce toxicity from subsequent doses has been well documented (MacNider, 1929; Honda and Sudo, 1987), but the mechanism responsible for this acquired resistance has been elusive. As reported previously (Mizuno et al., 1997), rats given a conditioning dose of 5 mg/kg uranyl acetate exhibited moderate nephrotoxicity with necrosis of tubular epithelium. Although less cellular injury was apparent in conditioned animals than in naïve animals, there was only a slight decrease in azotemia (Table 1). This is similar to previous work with dichlorovinylcysteine (DCVC) which demonstrated that prior exposure to DCVC or mercury could prevent lethality from subsequent DCVC exposure but did not affect markers of renal function (Vaidya et al., 2003). While necrosis is a prominent feature of uranium nephrotoxicity, significant numbers of tubular epithelial cells undergoing apoptosis were also observed. Uranium-induced apoptosis has been reported in macrophages (Kalinich et al., 2002), but this is the first report of its occurrence in kidney.

The protection provided by the conditioning dose was variable among animals. In some cases the Conditioned animals showed marked protection and in other cases the response was similar to the Unconditioned animals. Previous studies have noted that significant renal toxicity is required before acquired resistance will develop. Honda et al. (1987) showed that an intravenous conditioning dose of 0.9 mg/kg uranyl acetate induced azotemia in 8 of 14 rabbits. When these rabbits were injected with a second, higher dose of uranyl acetate (2 mg/kg, i.v.), only the rabbits that experienced azotemia from the conditioning dose were protected from development of non-oliguric renal failure. In the present study, the conditioning dose of uranyl acetate (5 mg/kg, i.p.) may not have been sufficient to produce acquired resistance in all of the animals. Because renal toxicity produces wasting in rats, weight gain was used to stratify the animals receiving the uranyl acetate conditioning dose into 'responders' and 'non-responders'. Weight gain in non-responders was similar to control animals, and BUN and serum creatinine levels were comparable to Unconditioned controls following a second uranyl acetate treatment. The responders gained less weight, suggesting that they experienced greater nephrotoxicity from the conditioning dose. These animals (i.e., the responders) exhibited significantly less toxicity from the second treatment with uranyl acetate.

Hsp induction has been proposed as a possible mechanism in acquired resistance to uranium. Upregulation of Hsps is associated with decreased susceptibility to toxicity from a variety of agents (Zager et al, 1994; Elliott et al., 1982), and kidney cells expressing elevated levels of Hsps in response to a conditioning uranyl acetate dose might be more resistant to toxicity from a subsequent dose. Previously, a conditioning dose of uranyl acetate was reported to result in increased levels of Hsp73 [Hsc70] in rat kidney homogenate (Mizumo et al., 1997). In the present study, uranyl acetate was observed to increase levels of Hsp25, Hsp32, and Hsp70i, but not Hsc70, in rat kidney homogenates at the end of the conditioning period. The reason for the differences in Hsps elevated in rats by uranyl acetate treatment in the two studies is unclear, but could be related to differences in dosing route or in the specificity of the antibody used for Hsp detection.

Immunostaining for Hsp25 and Hsp70i demonstrated prominent expression of these proteins in tubular epithelial cells. Many cells that highly expressed these Hsps had morphological characteristics of regenerating tubular epithelial cells. Immunostaining for PCNA positive cells confirmed the presence of regenerating cells in these sections (Figures 1C and 1D). While we were not able to perform double label experiments on these sections, the morphological characteristics and distribution of PCNA positive and Hsp positive cells is comparable and indicates that regenerating cells are expressing high levels of Hsps. Previous work demonstrated that ischemia also markedly induces Hsp25 and Hsp70i in cortical tubular epithelium (Smoyer et al., 2000). However, ischemia induced these proteins in cortical tubules and glomeruli within 6 hours of insult, suggesting that there are mechanistic differences in the hsp response to uranium and ischemia. Although a detailed time course of the Hsp response to uranyl acetate was not conducted, no increase was observed 24 hours after a nephrotoxic uranyl acetate dose (10 mg/kg, i.p.), despite degenerative changes and the appearance of necrosis in tubular epithelium. In this respect, the induction of Hsps in response to uranyl acetate is different from other metals such as cadmium, where increased Hsp expression precedes the development of frank renal injury (Goering et al., 2000). In the report by Mizuno (1997), Hsp levels began to increase at 3 days after a uranyl acetate dose and diminish by 4 weeks, which correlates well with the onset and completion of tubular regeneration (Haley et al., 1982). Increases in Hsps have been reported to occur in regenerating cells, including regenerating tubular epithelium (Zhu et al., 1996; Morita et al., 1995). Thus, increases in kidney Hsps following a conditioning dose of uranyl acetate appear to be a function of epithelial repair rather than a specific response to uranyl acetate. Given the apparent requirement that the conditioning uranyl acetate dose produce some nephrotoxicity to be protective, as discussed above, the link between regenerating epithelium and Hsp induction explains why Hsp increases are correlated with acquired resistance. It does not, however, constitute direct evidence that Hsps are responsible for the protection.

Additional insight was sought in experiments using renal tubular epithelial cells (RK3E and LLC-PK1 cells) in culture. Dramatic induction of Hsp25 and Hsp70i was observed eighteen hours after the classical Hsp inducer, mild heat shock (42° C for 30 minutes). Although the cells were clearly capable of mounting an Hsp response, no increase in Hsps was observed if cells were treated instead with uranyl acetate, including cytotoxic concentrations. This is in agreement with the concept from *in vivo* studies that uranyl acetate cytotoxicity does not directly increase Hsps. Induction of Hsps by most toxic compounds is thought to be caused by the presence of misfolded proteins (Ananthan et al., 1986), and the lack of response to uranium may indicate that proteotoxicity is not prominent in uranyl acetate-induced renal injury.

A previous study found that heat stress and prior exposure to UA resulted in reduced toxicity from subsequent uranium exposure in LLC-PK1 porcine kidney cells (Furuya et al., 1997). In this study, treatment with uranium occurred 5-6 days after heat stress, and the time course of Hsp induction was not characterized. Consequently, it is unclear whether Hsps remained elevated at the time of the uranium exposure. In the present study, uranyl acetate was added to cultures of heat-stressed RK3E and LLC-PK1 cells when Hsps were strongly upregulated. No effect on uranyl acetate cytotoxicity was observed. This is consistent with the observation that thermal pretreatment of rats failed to alter uranyl acetate toxicity (Mizuno et al., 1997). The authors of that study interpreted the lack of effect from thermal pretreatment, which did not affect Hsc70, as confirmation of a role for Hsc70 in acquired resistance. An alternative explanation is that elevated Hsps do not offer protection from uranyl acetate toxicity. In the present study, prior exposure of LLC-PK1 cells to UA did reduce toxicity of subsequent exposures, however, neither Hsp70i or Hsp25 expression was elevated at the time of exposure. Our in vitro data strongly suggests that protection provided by prior exposure to heat stress or UA is not due to elevated levels of Hsp but some other process such as selection for a resistant cell population.

As the preceding discussion indicates, establishing the role for Hsps in uranium acquired resistance is confounded somewhat by apparently conflicting observations. However, experimental results presented here and elsewhere are in agreement in indicating that moderate renal injury from a conditioning dose is required to produce acquired resistance from uranium. The present study suggests that increased Hsps in kidney are a consequence of cellular regeneration in response to this toxicity, but are not directly cytoprotective. Cell proliferation has also been linked to resistance from other nephrotoxic compounds such as dichlorovinyl-*L*-cysteine and mercury (Vaidya et al., 2003). Better understanding of the mechanisms that renders proliferating cells resistant to toxicity may clarify uranium acquired resistance and provide insight into interventional strategies for renal failure in general.

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Table 1. Biochemical and histological parameters of acquired resistance from uranyl acetate (uranyl acetate) nephrotoxicity in rat.

	<u>**Control**</u>	<u>**Unconditioned**</u>	<u>**Conditioned**</u>
Conditioning treatment	**Saline**	**Saline**	**uranyl acetate (5mg/kg)**
Challenge treatment	**Saline**	**uranyl acetate (10mg/kg)**	**uranyl acetate (10mg/kg)**
Blood Urea Nitrogen (mg/dL)	46 ± 12	283 ± 38*a*	226 ± 62*a*
Serum Creatinine (mg/dL)	63 ± 5	498 ± 45*a*	384 ± 71*a*
Histology Score (1-5)	1 (1-1)	4 (3-4)*a*	2 (2-3)*a,b*

Rats were conditioned with either saline (control) or uranyl acetate (5 mg/kg, i.p.) and treated 10 days later with either saline or uranyl acetate (10 mg/kg, i.p.). Serum creatinine and blood urea nitrogen were measured in serum collected 5 days after the second treatment. Histopathological scores were evaluated in H&E stained kidney sections. Scores based on extent of tubular injury and ranged from 1 (normal) to 5 (severely affected). Values are presented as mean ± SE (n=9) for serum parameters and median (range) for histology scores. *a* Indicates values significantly different than control (saline – saline). *b* Indicates values significantly different from Unconditioned rats.

Figure Legends

Figure 1. Photomicrographs of of rat kidney sections from control (saline) and Conditioned animals 10 days after treatment with the conditioning dose of 5 mg/kg uranyl acetate. A) H&E stained sections of control rats. Normal tubule is indicated by arrow. B) H&E stained section of UA conditioned rat. Regenerating tubular epithelium is evident as flattened, basophilic staining epithelial cells indicated with arrow. C) Immunohistochemical staining for PCNA in kidney sections from control rats. D) Immunohistochemical staining for PCNA in kidney sections from UA conditioned rat. Nuclei in cells in late S phase stain darkly and are indicated by arrow.

Figure 2. Heat shock protein (Hsp) induction in rat kidney 10 days after an acute dose of uranium acetate (5 mg/kg i.p.). Kidney homogenate was prepared as described in Methods and the proteins were resolved by SDS-PAGE. The level of each Hsp was measured by Western blotting employing an antibody that specifically recognizes one of the following Hsps: Hsp25, Hsp32, Hsc70, or Hsp70i.

Figures 3 (A-D). Immunohistochemical detection of heat shock protein (Hsp) induction in rat kidney 10 days after a conditioning dose of uranyl acetate (5 mg/kg, i.p.). Kidneys were fixed in formalin, embedded in paraffin, sectioned, and probed with an antibody that recognizes either Hsp25 or Hsp70i. Kidney sections were: a) Saline treated control – anti-Hsp25; b) uranyl acetate (5 mg/kg, i.p.) pretreated – anti-Hsp25 c) Saline treated control – anti-Hsp70i; d) uranyl acetate (5 mg/kg, i.p.) pretreated – anti-Hsp70i. Arrows highlight intense staining in flattened regenerating tubular epithelium. Immunostaining is shown in red.

Figure 4 (A-C). Photomicrographs of H&E stained sections of rat kidney from control, Unconditioned and Conditioned animals 5 days after treatment with 10 mg/kg uranyl acetate.

Unconditioned animals exhibit severe tubular degeneration while regenerating epithelium in conditioned animals is spared.

Figure 5. Effect of uranyl acetate treatment on Hsp expression. A) Expression of Hsp70i, Hsc70, and Hsp25 in rat kidney 24 hours after treatment with 10 mg/kg uranyl acetate. B) Expression of Hsp25 and Hsp70i in RK3E rat kidney epithelial cells 24 hours after indicated treatment. C) Expression of Hsp70i in LLC-PK1 porcine kidney epithelial cells 24 hours or 6 days after uranyl acetate pretreatment. Ctrl – untreated cells ; Heat – 24 hours after exposure to 42°C for 30 min.

Figure 6. Cytotoxicity of uranyl acetate in rat kidney epithelial cells. Heated cells were exposed to heat (42°C for 30 minutes) 24 hours prior to uranium treatment. They were then treated with various concentrations of UA and viability determined by XTT assay 24 hours after UA treatment. UA concentrations 500μM and greater significantly reduced viability compared to their respective controls ($p<0.05$), however there were no differences between control and heated cells at a given UA concentration. Values are mean ± SD (n=3). A) UA toxicity in RK3E cells B) UA toxicity in LLC-PK1 cells.

Figure 7. Prior exposure to UA reduces subsequent toxicity of UA in LLC-PK1 cells. Pretreated cells were treated with 1mM UA for 24 hours. Cells were then washed and replated. When confluent, cells were treated with indicated concentrations of UA and LDH release was measured 48 hours later. Values are mean ± SD, (n=3). All UA concentrations tested significantly increased LDH release compared to their respective controls ($p<0.05$). * denotes values in UA pretreated cells are significantly different than non-pretreated controls ($p<0.05$).

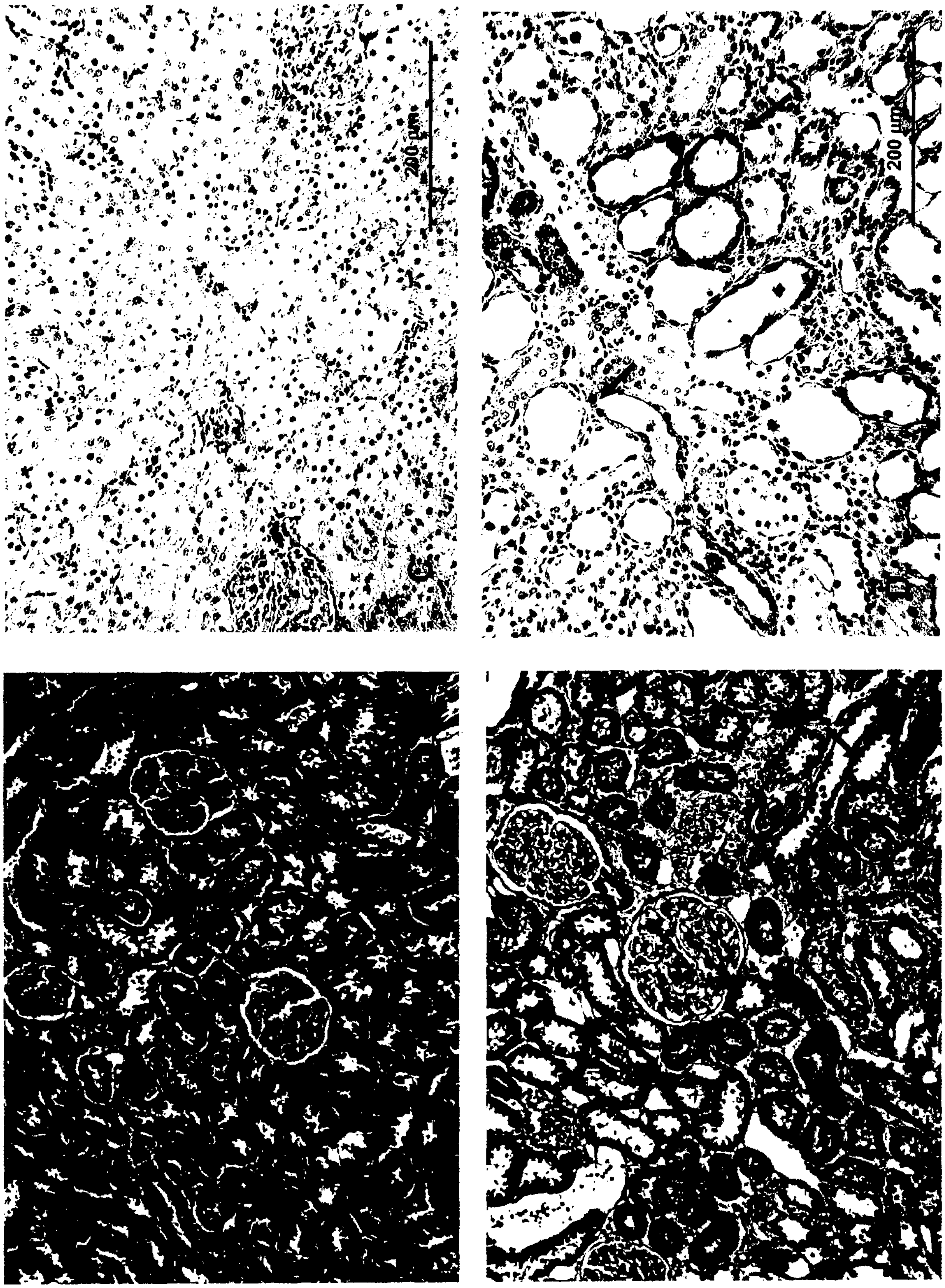
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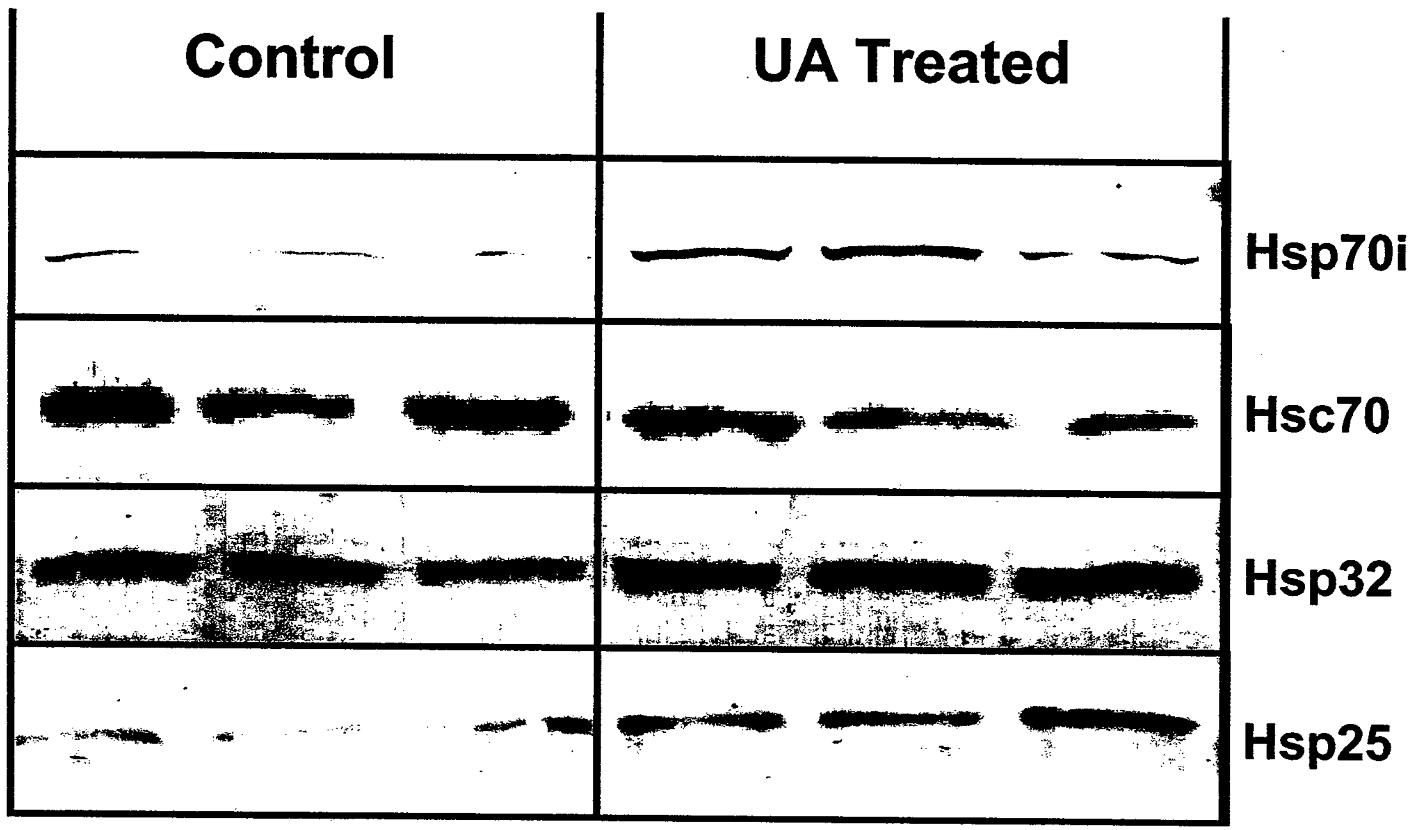
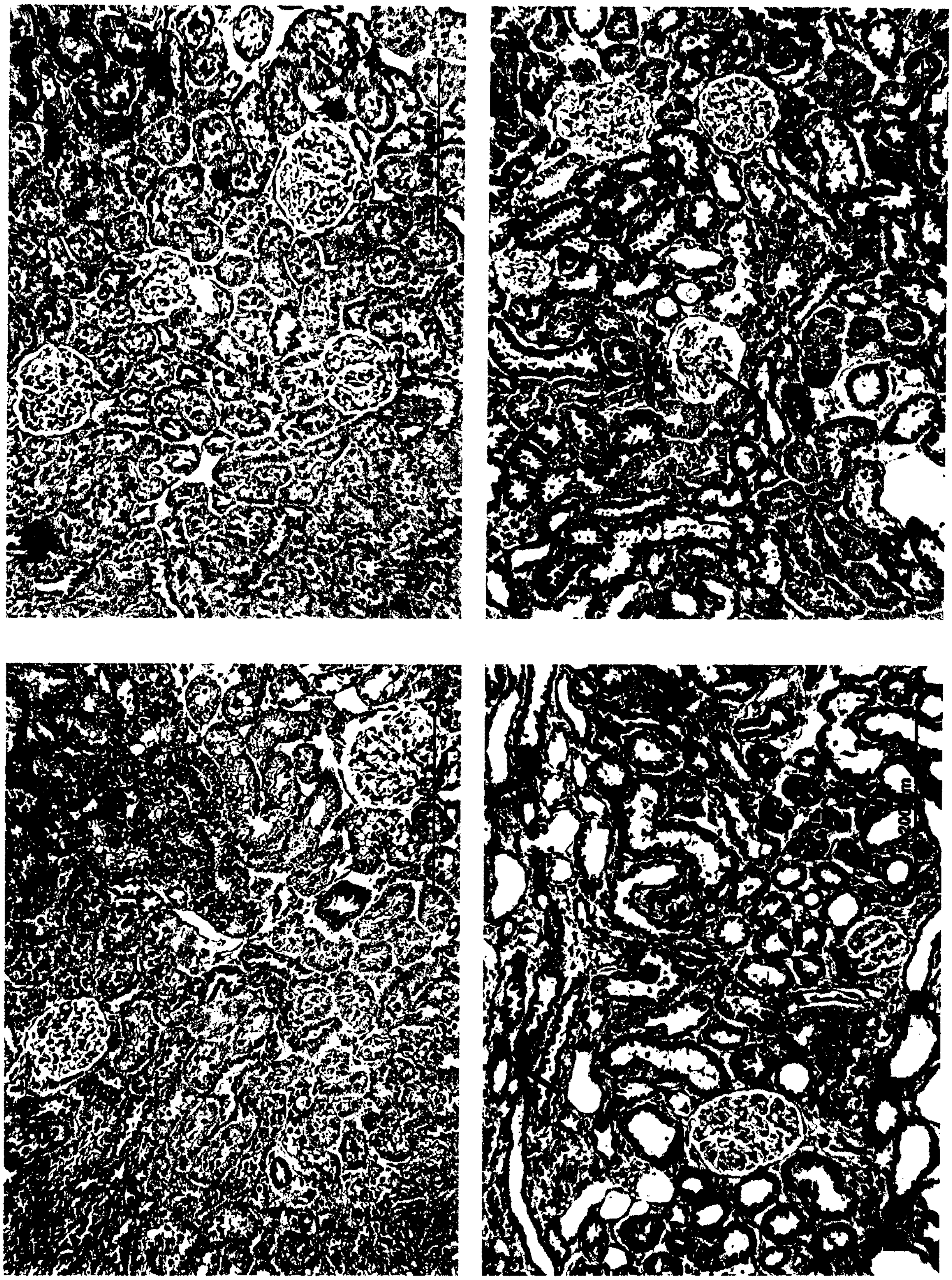
Figure 2





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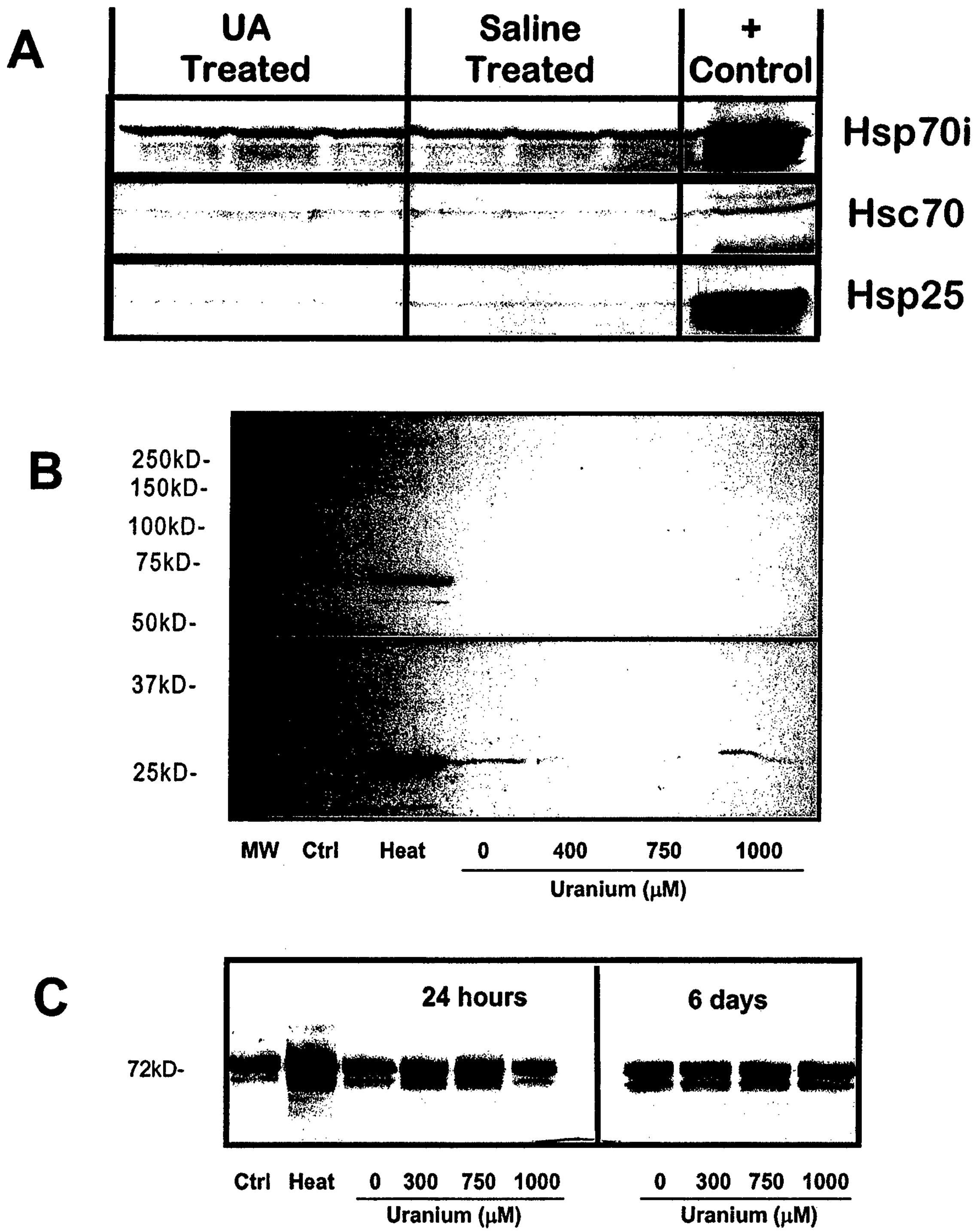
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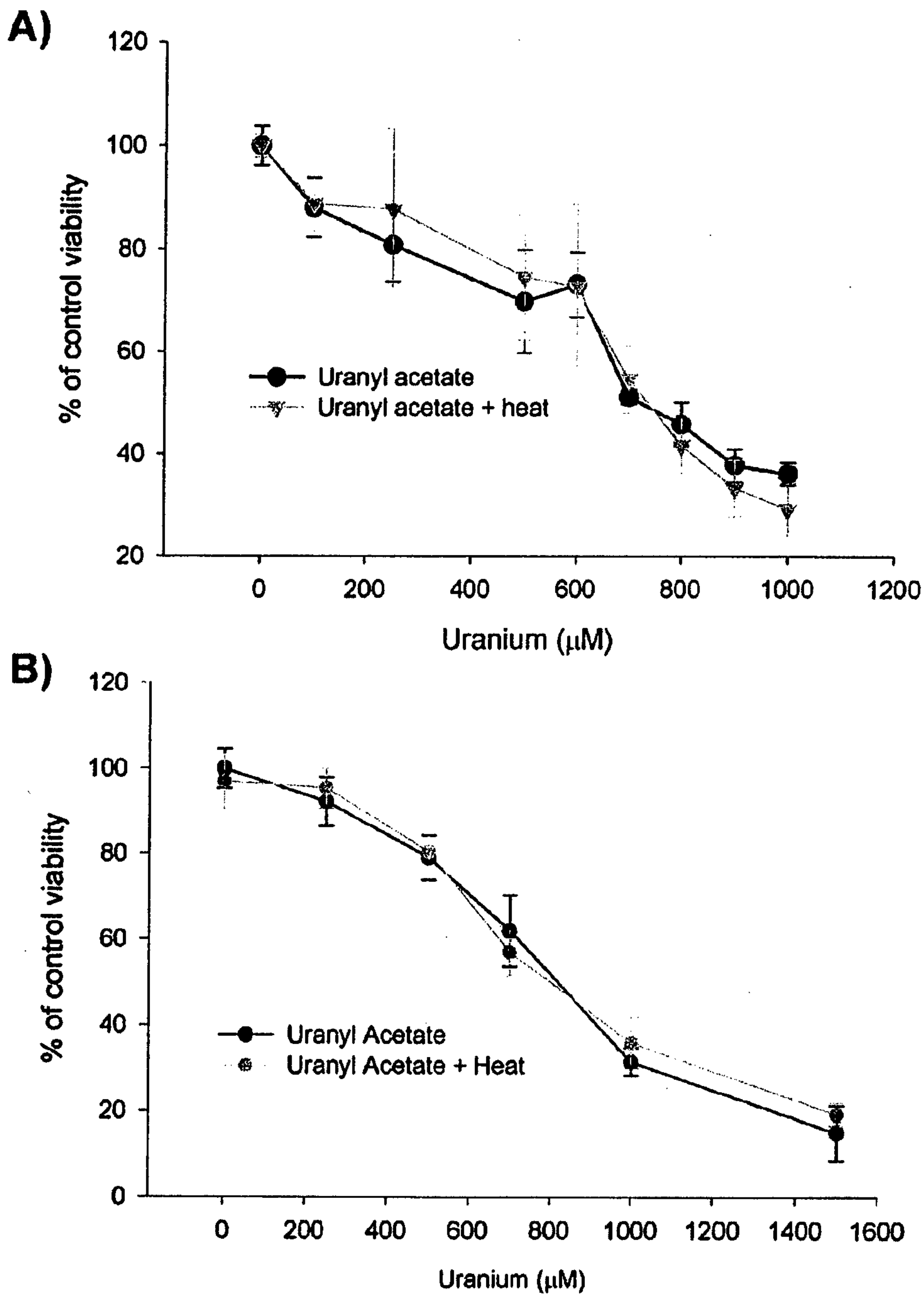
Figure 6


A)
% of control viability
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Uranyl acetate + heat
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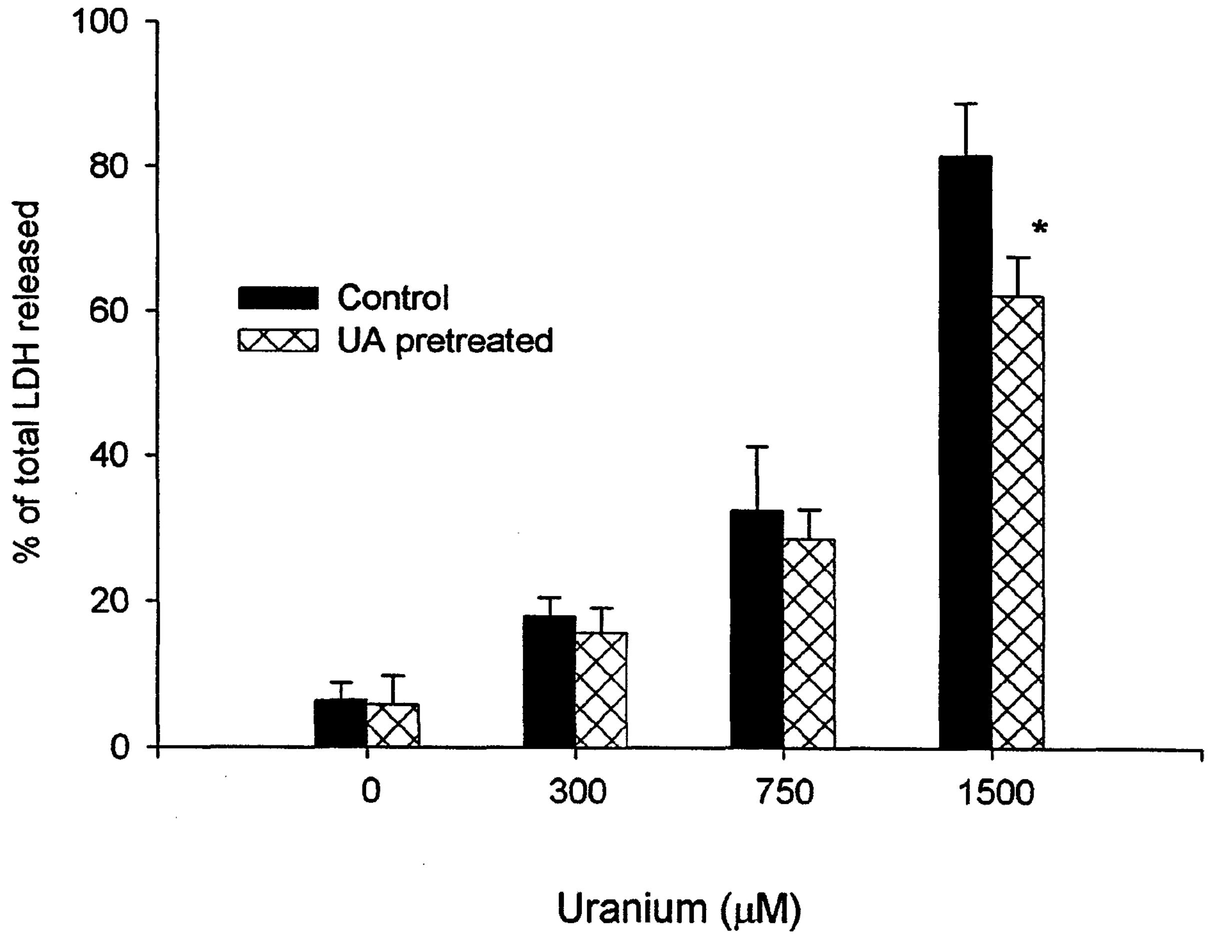
B)
% of control viability
Uranyl Acetate
Uranyl Acetate + Heat
Uranium (µM)

Figure 7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